Monitoring of Fungal Growth and Degradation of Wood

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Academic Dissertation in Microbiology

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Cover photo:
Brown-rotted timber from an old wooden cabin in Polvijärvi

Contents

List of original publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals (I-IV). In addition, unpublished data are also presented.

I Vares, T., **Niemenmaa, O**. and Hatakka, A. 1994. Secretion of ligninolytic enzymes and mineralization of ^{14}C-ring-labelled synthetic lignin by three *Phlebia tremellosa* strains. Applied and Environmental Microbiology 60:569-575

II **Niemenmaa, O**., Uusi-Rauva, A. and Hatakka, A. 2006. Wood stimulates the demethoxylation of [O^{14}CH$_3$]-labelled lignin model compounds by the white-rot fungi *Phanerochaete chrysosporium* and *Phlebia radiata.* Archives of Microbiology 185:307-315

III **Niemenmaa, O**., Uusi-Rauva, A. and Hatakka, A. 2008. Demethoxylation of [O^{14}CH$_3$]-labelled lignin model compounds by the brown-rot fungi *Gloeophyllum trabeum* and *Poria (Postia) placenta.* Biodegradation DOI: 10.1007/s10532-007-9161-3.

IV **Niemenmaa, O**., Galkin, S., Hatakka, A. 2008. Ergosterol contents of some wood-rotting basidiomycete fungi grown in liquid and solid culture conditions. International Biodeterioration & Biodegradation doi:10.1016/j.ibiod.2007.12.009

The author's contribution

I Outi Niemenmaa planned the experiments together with Tamara Vares, and did the laboratory work together with her: culturing of fungi, the enzyme assays from the fungal liquid cultures, enzyme purification with anion-exchange chromatography and characterization of the enzyme with SDS-PAGE and IEF. Outi Niemenmaa conducted the mineralization experiments of ^{14}C-(ring)-labelled synthetic lignin (DHP). Outi Niemenmaa interpreted the results with Tamara Vares.

II-IV Outi Niemenmaa planned the experiments and did the laboratory work, except the ergosterol analysis by HPLC, interpreted the results, analysed the data and wrote the articles.

Abbreviations

AAO	aryl alcohol oxidase (EC 1.1.3.7)
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADMS	asparagine ammonium nitrate dimethylsuccinate medium
ATP	adenosine triphosphate
CBQ	cellobiose:quinone oxidoreductase (EC 1.1.5.1)
CO_2	carbon dioxide
DHP	dehydrogenation polymerizate, synthetic lignin
DMF	dimethyl formamide
dw	dry weight
EG	endo-1,4-β-glucanase (EC 3.2.1.4)
FPLC	fast protein liquid chromatography
FT-NIR	Fourier transform near-infrared (spectroscopic technique)
GC-MS	gas chromatography - mass spectrometry
GLOX	glyoxal oxidase (EC 1.2.3.5)
HBT	1-hydroxybenzotriazole
HC	high glucose (1.0%)
HN	high-nutrient nitrogen medium, containing 20 mM as nitrogen (N)
H_2O_2	hydogen peroxide
HPLC	high-performance liquid chromatography
IEF	isoelectric focusing
kDa	kiloDalton, 10^3 Da
LC	low glucose (0.1%)
LiP	lignin peroxidase (EC 1.11.1.14)
LN	low-nutrient nitrogen medium, containing 2 mM as nitrogen (N)
MnP	manganese peroxidase (EC 1.11.1.13)
MW	molecular weight
MeOH	methanol
ODC	oxalate decarboxylase (EC 4.1.1.2)
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
p*I*	isoelectric point
PLFAs	phospholipid fatty acids
SDS	sodium dodecyl sulphate
Tween 80	sorbitan polyoxyethylene monooleate
VA	veratryl alcohol
VPs	versatile peroxidases

Abstract

In the present work the growth and activities of some basidiomycetous fungi were studied. Three approaches were used: measurements of extracellular enzyme activities (lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase), radioisotopic assays based on ^{14}C-(ring)-labelled synthetic lignin (DHP), [O^{14}CH$_3$]-labelled nonphenolic β-O-4 dimer (veratrylglycerol-β-guaiacyl ether) and [O^{14}CH$_3$]-labelled vanillic acid, and ergosterol analysis. The radioisotopic method is one of the most reliable techniques in determining the growth and activities of fungi. Measurement of enzyme activity is another way of evaluating the biological activity of a fungus, which in turn is related to the fungal growth. Ergosterol is a good indicator of fungal growth because it is the main sterol in fungal cell membranes and is almost exclusively present in living fungi.

The white-rot fungal strains of *Phlebia radiata* and *P. tremellosa* were similar, based on enzyme activities. A total of 2–3 LiPs were purified from all these strains as well as two MnPs from the *P. radiata* strains. All four *P. tremellosa* strains showed MnP activity, but the enzyme could not be purified. *Phlebia radiata and P. tremellosa* strains also showed laccase activity.

The presence of wood in the medium increased the evolution of the ^{14}CO$_2$ from [O^{14}CH$_3$]-labelled β-O-4 dimer by the white-rot fungi *P. radiata* and *Phanerochaete chrysosporium* and the brown-rot fungus *Gloeophyllum trabeum* especially in an air atmosphere with no nutrient supplements (nitrogen or glucose). Wood also in most cases compensated for nitrogen and glucose in wood-agar cultures by fungi in both atmospheres, i.e. wood supplied nutrients to the fungi for efficient demethoxylation. An air atmosphere was at least as good as an oxygen atmosphere for demethoxylation activity of the fungi when they were grown in ^{14}C-labelled cultures with wood blocks.

The highest amount of biomass, based on an ergosterol assay, was obtained by *P. radiata* and *P. chrysosporium* in low-nutrient nitrogen (2 mM-N) medium from wood in solid agar cultures. The medium combination 2 mM-N with low glucose (0.1%) was chosen for experiments in liquid cultures, from which the ergosterol contents of six fungi were analysed weekly during five weeks. Under the same conditions as used for the white-rot fungi, the brown-rot fungi *G. trabeum* and *Poria* (*Postia*) *placenta* grew well and they indicated the highest ergosterol contents.

Phlebia radiata was used in all cultures and experiments in which different methods were tested. The results showed that the lignin peroxidase activities and the radioisotopic method (the evolution of ^{14}CO$_2$ when ^{14}C-labelled compounds were used as substrates) correlated very strongly. The ergosterol content also correlated well with LiP activity, but to verify the true significance of this, samples should be taken almost daily during the early stages of growth.

Tiivistelmä (Abstract in Finnish)

Valko- ja ruskolahosienet tunnetaan luonnossa puun ja karikkeen lignoselluloosan lahottajina. Valkolahosienet pystyvät hajottamaan kaikkia puun osia: ligniiniä, selluloosaa ja hemiselluloosaa. Selektiivisesti ligniiniä hajottavat sienet lahottavat puusta suhteessa enemmän vaikeasti hajoavaa ligniiniä kuin selluloosaa tai hemiselluloosaa, jolloin jäljelle jää valkoista ja miltei puhdasta selluloosaa. Bioteknisissä sovelluksissa juuri selektiiviset valkolahottajat ovat kiinnostavia. Niiden avulla voidaan puuhaketta esikäsitellä esimerkiksi paperinvalmistuksessa haitallisen ligniinin poistamiseksi.

Ruskolahosienet ovat huomattavia puun, puutavaran ja puisten rakenteiden lahottajia, kuten tässä työssä käytetty *Gloeophyllum trabeum* (saunasieni) ja *Poria (Postia) placenta* (istukkakääpä). Ruskolahosienet hajottavat puusta hemiselluloosan lisäksi selluloosaa, jolloin jää jäljelle ruskea, kuutiomaiseksi kutistunut ja jauhomaiseksi mureneva ligniini. Ruskolahosienet irrottavat metyyliryhmiä ligniinin aromaattisista renkaista ja muovaavat siten ligniiniä jonkin verran.

Kahden ruskolahosienen *G. trabeum*in ja *P. placenta*n lisäksi tutkittiin valkolahosieniä, joista *Ceriporiopsis subvermispora* (karstakääpä) ja paloalueelta eristetty harvinainen *Physisporinus rivulosus* -sieni (talikääpä) hajottavat ligniiniä erittäin selektiivisesti. *Phanerochaete chrysosporium* on kaikkialla paljon tutkittu sieni, ja *Phlebia radiata* valkolahosientä (rusorypykkä) on tutkittu paljon mikrobiologian osastolla. Lisäksi selvitettiin *Phlebia tremellosa* -sienten (hytyrypykkä) ligninolyyttisten entsyymien tuottoa ja ^{14}C-leimatun synteettisen ligniinin (DHP) hajotusta. *P. tremellosa* -sienillä tutkittiin 10 ja 50 kertaisten mangaanimäärien vaikutusta kasvatusalustoissa normaalipitoisuuteen verrattuna. *P. radiata* ja *P. tremellosa* -sienten on todettu useissa tutkimuksissa hajottavan ligniiniä selektiivisesti.

Tutkimuksessa selvitettiin miten sienten kasvua voi mitata, miten vertailukelpoisia eri mittaamismenetelmillä saadut tulokset ovat ja ilmenevätkö sienten aktiivisimmat kasvuvaiheet samaan aikaan eri menetelmillä mitattuna. Sienten kasvatusalustoilta määritettiin solunulkoisten ligninolyyttisten entsyymien aktiivisuudet (ligniini- ja mangaaniperoksidaasi, ja lakkaasi; *P. tremellosa, P. radiata*), demetoksyloituminen [O^{14}CH$_3$]-leimatusta malliyhdisteestä (β-O-4 dimeeri; *P. chrysosporium, P. radiata, G. trabeum, P. placenta*), ja puun vaikutusta hajotukseen, sekä mineralisoituminen synteettisestä ^{14}C-rengas leimatusta ligniinistä (DHP) (*P. radiata*, kolme *P. tremellosa* -kantaa). Sienten biomassa (kuivapaino ja ergosteroli) määritettiin ruskolahosienten (*G. trabeum, P. placenta*) sekä *P. radiata*n ja *P. chrysosporium*in lisäksi *P. rivulosus* - ja *C. subvermispora* -sienistä. Määritykset tehtiin nesteviljelmistä ja kiinteällä agaralustalla puun kanssa ja ilman puuta tehdyistä kasvatuksista viiden viikon aikana. Isotooppikokeissa tutkittiin erilaisia ravinnepitoisuuksia (typpi, glukoosi) sekä normaalin ilma-atmosfäärin lisäksi happiympäristön vaikutusta tuloksiin.

Tärkeimmät tulokset olivat seuraavat havainnot: (i) *P. radiata* ja *P. tremellosa* -sienikannat tuottivat ligniini- ja mangaaniperoksidaasia sekä lakkaasia, ja kaikista puhdistettiin 2-3 ligniiniperoksidaasientsyymiä ja *P. radiata* -sienistä yksi mangaaniperoksidaasi; (ii) kolme *P. tremellosa* -sienikantaa mineralisoivat rengasleimattua DHP:tä yhtä hyvin kuin *P. chrysosporium* ja *P. radiata*; (iii) korkeat mangaanipitoisuudet kasvualustassa ehkäisivät mineralisaatiota, mikä vahvistaa ligniiniperoksidaasientsyymin merkitystä ligniinin hajoamisessa; (iv) puu kasvatusalustassa lisäsi valkolahosienten (*P. radiata* ja *P. chrysosporium*) ja ruskolahosienen (*G. trabeum*) demetoksylaatiota

useimmissa alustavaihtoehdoissa, eli $^{14}CO_2$:n tuottoa metoksyylissä leimatusta ligniinin malliyhdisteestä kiinteään agaralustaan verrattuna erityisesti ilma-atmosfäärissä; (v) hapessa paras demetoksylaatio ($^{14}CO_2$:n tuotto) saatiin puupalakasvatuksissa, joihin oli lisätty ravinnetyppeä tai typen lisäksi glukoosia sekä valkolahosienillä että *G. trabeum* -ruskolahosienellä; (vi) ilmassa demetoksylaatio oli puulla voimakkainta valkolahosienillä ilman ravinnelisäyksiä kun taas *G. trabeum* -sienellä se oli yhtä hyvä eri alustavaihtoehdoissa; (vii) biomassan muodostuminen rihmastojen ergosterolipitoisuuksien avulla mitattuna oli ruskolahosienillä parempi kuin valkolahosienillä; (viii) ja biomassojen huippupitoisuudet olivat kuudella sienellä eri suuruisia ja niiden maksimimäärien ajankohdat vaihtelivat viiden viikon kasvatuksen kuluessa.

Mikrobiologian osastolla eristetty ja paljon tutkittu *Phlebia radiata* -valkolahosieni oli mukana kaikissa tehdyissä kokeissa. Tämän sienen ligniiniperoksidaasiaktiivisuus ja $^{14}CO_2$:n tuotto ^{14}C-rengas-leimatusta synteettisestä ligniinistä (DHP) korreloivat erittäin hyvin keskenään. Biomassan muodostuminen ergosterolilla määritettynä tuki hyvin entsyymiaktiivisuusmittauksilla ja isotooppikasvatuksilla saatuja tuloksia.

1 INTRODUCTION

1.1 Wood, lignocelluloses and lignin

1.1.1 Lignocellulosic biomass

The major components in wood are cellulose, hemicellulose and lignin. Woody and dead agricultural plant materials constitute more than 60% of the total biomass produced on the earth. The degradation of lignocellulosic materials is considered the most important of biological processes, leading to production of carbon dioxide, water and humic substances in the earth's carbon cycle (Kuhad *et al.* 1997). Concern over food and energy sufficiency became apparent for more than 30 years ago, due to the first so-called oil crisis of 1973. Interest in the use of abundant renewable lignocellulose as an energy source has again been kindled worldwide. Lignocellulosic biomasses, such as agricultural and forestry residues, were recognized as a potential source of energy that could replace the future transport of unstable and uncertain petroleum and fossil fuel (Himmel *et al.* 2007). The most common renewable fuel is ethanol, which could be derived from lignocellulosic material, cellulose and hemicellulose, as an attractive future raw material (Wyman 2003; Gray *et al.* 2006). In many countries there are research and development programmes in renewable energy sources; e.g. the European Union has envisions a future in which one fourth of fuel will be derived from biomaterials by 2030 (Mosier *et al.* 2005; Biofuels in the European Union, A Vision for 2030 and beyond 2006) and in Finland there are plans to take lignocelluloses in to use more than ever before (Helsingin Sanomat 2007a; b). The goal in this research is to alter and improve lignocellulosic material with pretreatments for industrial use; e.g. to increase the yields of fermentable sugars from cellulose and hemicellulose (Mosier *et al.* 2005). Another major application would be breaking the lignin and the crystalline cellulose structure with enzyme treatments in the pulp and paper industry, lower the costs of energy and chemicals, and improve efficiency (Martínez 2002; Mosier *et al.* 2005).

1.1.2 Cellulose

The main structural component in the cell wall of green plants is cellulose. Cellulose molecules form linear homopolysaccharide chains of β-D-glucopyranose units, which are joined together by β(1–>4)-glycosidic bonds. Cellulose molecules are strongly associated through inter- and intramolecular hydrogen bonds and bundles of cellulose molecules form microfibrils. They are organized in to strong fibrous structures, in which the highly oriented regions are called crystalline cellulose and the less oriented region amorphous cellulose. Most of the plant cell wall cellulose is crystalline. Cellulose microfibrils are embedded in a lignin-hemicellulose matrix (Sjöström 1993; Kuhad *et al.* 1997; Teeri 1997; Daniel 2003).

1.1.3 Hemicellulose

Hemicelluloses are short chains of frequently branched heteropolysaccharides, containing two or more different sugar monomers, both hexoses and pentoses. Hemicelluloses are named after their main sugar constituents, such as galactoglucomannans, which are the principal hemicelluloses in softwoods and which are formed of galactose, glucose and mannose units. Glucuronoxylans predominate in hemicelluloses of hardwood, and are composed of β-D-xylose and 4-O-methyl-α-D-glucopyranosyluronic acid units. Hemicelluloses and lignin give the cell structure rigidity (Sjöström 1993; Kuhad *et al.* 1997).

1.1.4 Lignin

Lignin is the second most abundant biopolymer next to cellulose in terrestrial vascular plants. Lignin is a highly branched, amorphous and water-insoluble high-molecular weight (MW) natural polymer built of phenyl propane units (Sjöström 1993; Argyropoulos and Menachem 1997; Kuhad *et al.* 1997). Lignin is closely associated with cellulose and hemicelluloses in plant cell walls and attaches to polysaccharide polymers in the cell walls both physically and chemically. It binds the fibrous cell walls together and this gives plants mechanical strength. Lignin resists chemical and enzymatic degradation and makes wood resistant against microbial attack. The phenylpropane units of lignin are linked together by different types of bonds (Fig. 1.1) (Eriksson *et al.* 1990; Argyropoulos and Menachem 1997; Kuhad *et al.* 1997). The most common bond is the β-aryl ether (*β*-O-4) linkage, which may account for 50% and 60% of the intermonomeric bonds in softwood lignin and hardwood lignin, respectively (Sjöström 1993).

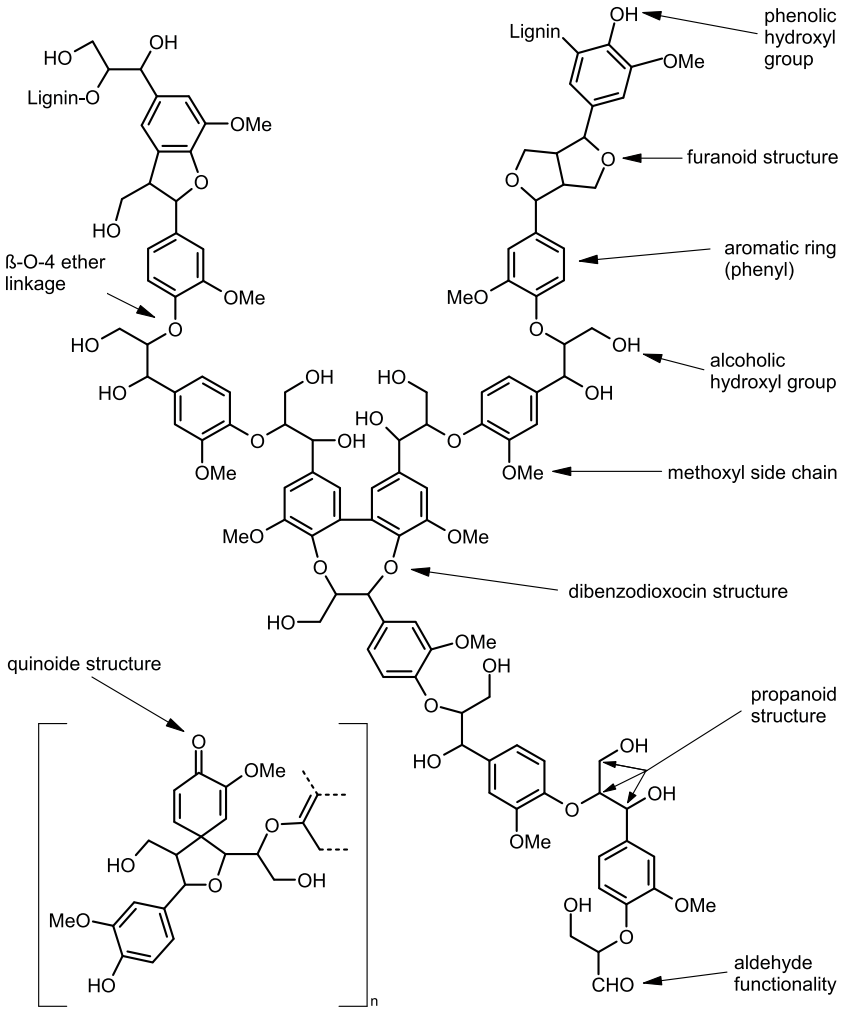
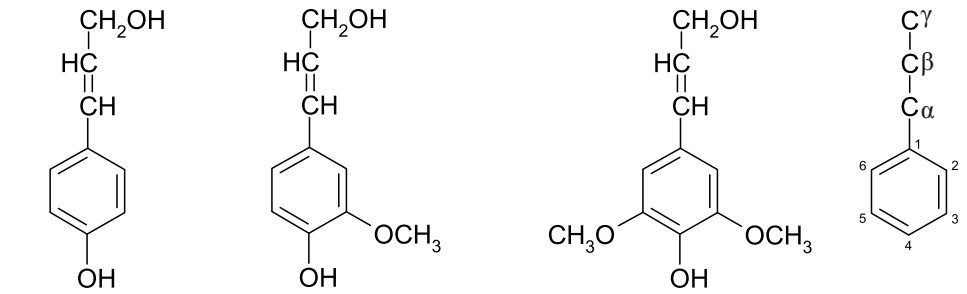


Fig. 1.1 Structural model of lignin by Brunow (2001).



p-coumaryl alcohol coniferyl alcohol sinapyl alcohol

Fig. 1.2 *p*-Hydroxycinnamyl alcohols, the precursors of lignin, and model for numeration and designation of the carbon skeleton in the lignin molecule, according to Buswell and Odier (1987).

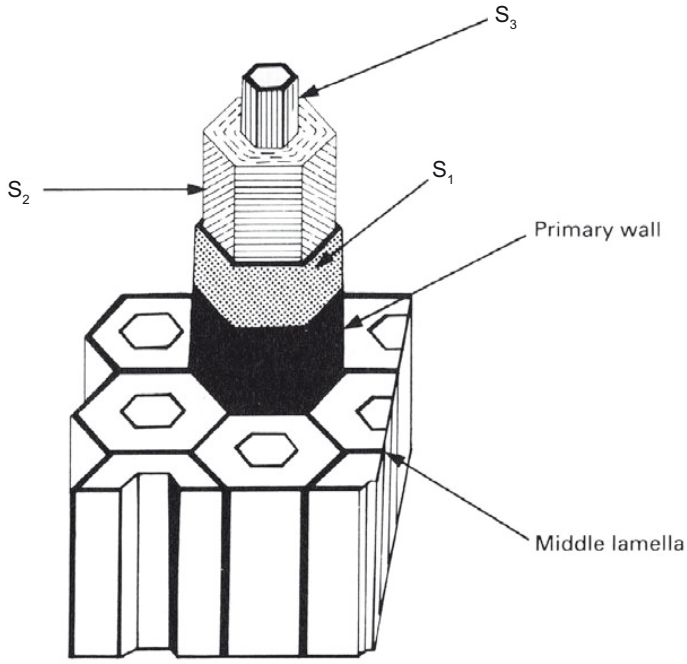


Fig. 1.3 Model of the cell wall structure of wood. S_1 = outer layer, S_2 = middle layer, S_3 = inner layer of secondary wall. Figure is reprinted from Dix and Webster (1995) with permission of Springer (earlier Chapman & Hall).

Lignin is synthesized with the fixation of CO_2 in plant cell walls and finally by the polymerization of radicals generated by the one-electron oxidation of lignin precursors (Kirk and Brunow 1988; Eriksson *et al.* 1990; Chiang 2006). The lignin monomeric precursors, *p*-hydroxycinnamyl alcohols, are synthesized from L-phenylalanine or L-tyrosine through the shikimic acid pathway. The intermediates are formed by deamination and several

successive hydroxylations and methylating with enzyme reactions, leading to *p*-coumaric, caffeic, ferulic, 5-hydroxyferulic and sinapic acid. Enzyme-mediated reductions create *p*-hydroxycinnamyl alcohols: coniferyl, sinapyl and coumaryl alcohol (Fig. 1.2), which are the primary precursors and building units of lignin. Of these coniferyl alcohol is the most common precursor (Kirk and Brunow 1988; Fengel and Wegener 1989; Eriksson *et al.* 1990; Sjöström 1993; Argyropoulos and Menachem 1997; Kuhad *et al.* 1997).

Softwood consists mainly of tracheids and ray cells that give strength to wood and provide water transport. Either bordered pits or large simple pits connect the softwood tracheids. Hardwood possesses an array of vessels, fibres and ray parenchyma cells that are connected by bordered or simple pits (Sjöström 1993; Kuhad *et al.* 1997). The woody cell wall is composed of secondary and primary walls (Fig. 1.3). The secondary wall has three distinct layers, thin outer (S_1) and inner layers (S_3) and a thick middle layer (S_2) (Sjöström 1993). Adjacent cells are separated by the pectin-rich middle lamella. The lignin content is highest in the S_2 layer because it is the thickest layer, but the greatest lignin concentration is in the middle lamella between cells (Sjöström 1993; Kuhad *et al.* 1997). Fungal hyphae penetrate from one cell to another through existing pores and pits in the cell walls (Eriksson *et al.* 1990).

1.1.5 Synthetic lignin DHP (dehydrogenative polymerizate)

Synthetic lignin, a dehydrogenative polymerizate (DHP) of coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol), is one of the most frequently used lignin model compounds in lignin biodegradation research. The lignin-like polymer is prepared by reacting coniferyl alcohol or other lignin precursors with peroxidases or other enzymes and hydrogen peroxide (Kirk and Brunow 1988). DHPs can be specifically labelled in the β- and γ-carbon of the propyl side chain, aromatic rings or in the methoxyl groups in DHP synthesis with a specifically labelled carbon atom of ^{14}C-coniferyl alcohol if it is desired to prepare a homopolymer (Haider and Trojanowski 1975; Kirk *et al.* 1975; Kirk and Brunow 1988). Faix *et al*. (1981) used all three precursors, namely coniferyl, *p*-coumaryl and sinapyl alcohols, in different proportions. The MWs of DHPs can vary from 1500 to 10 000, depending on the synthesis method used (Eriksson *et al.* 1990). DHPs are insoluble in water, and their solubility in organic solvent, e.g. dimethylformamide (DMF), is dependent on the preparation method (Kirk *et al.* 1975; Crawford 1981). DHPs are useful lignin model polymers, because their spectroscopic and chemical properties have been well characterized, and they contain the same intermonomer linkages found in natural lignin (Kirk 1975; Kirk and Brunow 1988). They resemble lignin in the cell walls of plants but they do not contain carbohydrates or proteins. Unfortunately, dimeric lignin model compounds and synthetic lignins are not commercially available (Haider and Trojanowski 1980; Eriksson *et al.* 1990).

Specifically ^{14}C-labelled natural lignins in lignocellulose *in situ* were originally used to monitor lignin biosynthesis. They are probably the most useful lignin substrates for microbial biodegradation and transformation research. Many important findings, which are involved in the physiology of the model white-rot fungus *Phanerochaete chrysosporium* Burds., were discovered using ^{14}C-labelled lignin (Keyser *et al.* 1978; Kirk and Farrell 1987; Hatakka 2001). The growing plant or fresh-cut twigs are labelled by feeding them with ^{14}C-labelled lignin precursor (L-phenylalanine, ferulic, cinnamic, *p*-coumaric or sinapic acid) and then allowing the radioactivity to incorporate into lignin (Haider and Trojanowski 1980; Buswell and Odier 1987; Eriksson *et al.* 1990). After feeding the material may be dried and ground, or ground under liquid nitrogen (Crawford 1981; Crawford and Crawford 1988).

1.2 Degradation of wood by fungi

1.2.1 Types of wood decay

Basidiomycetous white-rot and brown-rot fungi are the most efficient degraders of wood among microorganisms in the environment. White-rot fungi attack all components of wood: cellulose, hemicellulose and lignin. Some of them preferentially degrade lignin (Blanchette 1984; 1988; Eriksson *et al.* 1990; Goodell *et al.* 1997; Kuhad *et al.* 1997; Hatakka 2001).

Brown-rot fungi belong to the same taxonomic group as the basidiomycetous white-rot fungi, but they utilize predominantly hemicellulose and cellulose in wood, leaving behind a chemically modified lignin residue. White-rot fungi colonize both hardwoods and softwoods, although they primarily attack hardwood, while brown-rot fungi attack softwood (Messner *et al.* 2003). Soft-rot-type decay is caused by *Ascomycetes* and mitosporic fungi, the latter earlier called *Deuteromycetes* or *Fungi imperfecti*. They decompose wood carbohydrates, but some can partially degrade lignin. The majority of soft-rot fungi can release $^{14}CO_2$ from the methoxyl groups of lignin and break down side chains and ring carbons of DHP (Haider and Trojanowski 1975; Eriksson *et al.* 1990; Kluczek-Turpeinen *et al.* 2003; Liers *et al.* 2006). Another group of common wood-inhabiting fungi are the blue-staining fungi, which are also associated with the appearance of damage in wood, but they do not affect wood strength (Daniel 2003). Some bacteria are known to destroy wood; e.g. *Pseudomonas* sp. was able to degrade lignin more than cellulose in softwood (Yang *et al.* 2007). Common wood-rotting fungi and their synonym names are listed in Table 1.1.

Table 1.1 Synonym names of some white-rot and brown-rot fungi

Fungus	**Synonyms**
Ceriporiopsis subvermispora (Pilát) Gilbn. et Ryv.	*Gelatoporia subvermispora, Poria subvermispora, Ceriporiopsis rivulosus*
Phanerochaete chrysosporium Burds (Kirk 1984)	*Chrysosporium lignorum* (Bergman and Nilsson 1966) *Sporotrichum pulverulentum* Novobranova (Eriksson *et al.* 1990)
Phlebia tremellosa (Schrad.:Fr.) Nakas et. Burds.	*Phlebia tremellosus, Merulius tremellosus*
Physisporinus rivulosus (Berk. & Curt.) Ryv.	*Polyporus rivulosus, Poria rivulosa, Poria albipellucida, Rigidoporus rivulosus, Ceriporiopsis rivulosa*, see Hakala *et al.* (2007)
Trametes versicolor Linn. Fr.	*Coriolus versicolor, Polyporus versicolor, Polystictus versicolor*
Fomitopsis pinicola (Sw.) P. Karst.	*Fomes pinicola*
Gloeophyllum trabeum (Pers. Fr.) Murrill	*Lenzites trabea*
Poria (*Postia*) *placenta* Fr.	*Poria monticola* Murr.
Mitosporic fungi	*Deuteromycetes* or *Fungi imperfecti*

1.2.2 White-rot fungi

White-rot and litter-decomposing basidiomycetous fungi are the only microorganisms, that are able to efficiently degrade all the components of plant cell walls, both carbohydrates and lignin. They cause two main white-rot types: simultaneous or nonselective and selective white-rot. In selective white-rot lignin and hemicellulose are lost preferentially and in nonselective white-rot, cellulose, hemicellulose and lignin are degraded more or less simultaneously (Blanchette *et al.* 1985; Messner *et al.* 2003). *Phanerochaete chrysosporium* and *Trametes* (syn. *Coriolus, Polyporus*) *versicolor* (L.) Lloyd (Cowling 1961; Russell *et al.* 1961) are typical nonselective rot-type fungi (Hatakka 2001). *Ceriporiopsis subvermispora* (Otjen *et al.* 1987; Srebotnik *et al.* 1994; Hakala *et al.* 2004; Fackler *et al.* 2006)*, Pycnoporus cinnabarinus* (Ander and Eriksson 1977; Eggert *et al.* 1996), *Pleurotus ostreatus* (Jacg.) P. Kumm. (Martínez *et al.* 1994), *Phlebia radiata* (Ander and Eriksson 1977), *Phlebia tremellosa* (syn. *Merulius tremellosus*) (Schrad.) Nakasone & Burds. (Ander and Eriksson 1977; Blanchette and Reid 1986; Eriksson *et al.* 1990; Fackler *et al.* 2006) and *Physisporinus rivulosus* (Berk. & M.A. Curtis) Ryvarden (Hakala *et al.* 2004; 2005; Hildén *et al.* 2007) represent the selective degraders of wood, that remove lignin preferentially to cellulose. They are the most promising fungi for biopulping (Eriksson *et al.* 1990; Hatakka 2001). *Ceriporiopsis subvermispora* and *P. rivulosus* are often studied for biopulping; cultivation on wood chips prior to mechanical pulping saves refining energy.

Hatakka (1994) suggested that many selectively lignin-degrading white-rot fungi do not produce LiP, but produce MnP. *Ceriporiopsis subvermispora* and *P. rivulosus* lack LiP, but they produce MnP and laccase (Srebotnik *et al.* 1994; Hatakka 2001; Hildén *et al.* 2007). Srebotnik *et al*. (1994) showed that *C. subvermispora* mineralized lignin as efficiently as *P. chrysosporium* in wood, although it lacks LiP and degrades nonphenolic lignin structures by one-electron-oxidation mechanisms. The mediators may act together with a laccase, i.e. laccase-mediator system, or with a MnP, e.g. MnP-lipid peroxidation system (Kirk and Farrell 1987; Hatakka 2001). MnP is the most significant ligninolytic enzyme produced by *C. subvermispora* and *P. rivulosus*. Laccase is produced by almost all white-rot fungi (Hatakka 1994; 2001). *Ceriporiopsis subvermispora* strains were, along with *P. tremellosa,* the most efficient fungi and already more efficient than *Phlebia brevispora* Nakasone and *P. radiata* in delignification after 3 days growth, as monitored by Fourier transform-near-infrared spectroscopy (FT-NIR) on spruce wood shavings during solid-state fermentations (Fackler *et al.* 2006).

The lignin-selective fungus *P. tremellosa* caused 60-85% degradation of the lignin with only a small loss of cellulose (Blanchette and Reid 1986). In their studies *P. tremellosa* may also have been less specific for lignin removal under certain growth conditions, and preferentially lignin-degrading white-rot fungi can produce simultaneous rot. Therefore, their categorization as selective and nonselective fungi is not always clear, because some white-rot fungi are capable of attacking both selectively and nonselectively in different parts of wood (Kuhad *et al.* 1997; Messner *et al.* 2003).

1.2.2.1 *Phanerochaete chrysosporium*

Phanerochaete chrysosporium Burds. is the most intensively studied wood-decaying white-rot fungus and efficiently degrades lignin (Ander and Eriksson 1977). Taxonomically it belongs to the Corticiaceae. It is a resupinate or crust fungus and is thermophilic, the optimum temperature being about 40 °C. LiP was first found in *P. chrysosporium* cultures by two research groups (Tien and Kirk 1983; Glenn *et al.* 1983). Depending on culture

conditions, it produces variable amounts of LiP and MnP isoenzymes, but not laccase, and is thus an atypical white-rot fungus (Hatakka 1994). The presence of 10 LiP-encoding genes, five MnP-encoding genes and the absence of laccase-encoding genes was verified when the entire genome of that fungus was sequenced (Martínez *et al.* 2004). Veratryl (3,4-dimethoxybenzyl) alcohol (VA) was the first low-MW compound found in the liquid culture of *P. chrysosporium* and may be associated with lignin degradation (Lundquist and Kirk 1978). Many biochemical and physiological reactions related to lignin degradation were first found in cultures of *P. chrysosoporium* and subsequently in cultures of other fungi.

1.2.2.2 *Phlebia spp.*

Phlebia radiata Fr. is among the most commonly studied white-rot fungi. As with *P. chrysosporium* it belongs to the Corticiaceae, grows as a resupinate and frequently occurs in the environment in Finland, but is mesophilic. *Phlebia radiata* strain 79 (American Type Culture Collection (ATCC) 64658) is an efficient degrader of various ^{14}C-lignins (Hatakka *et al.* 1983), was studied under various culture conditions (Hatakka *et al.* 1987; 1989; Kantelinen *et al.* 1988; 1989; Niku-Paavola *et al.* 1990; Vares *et al.* 1995; Moilanen *et al.* 1996) and its extracellular enzymes described (Niku-Paavola *et al.* 1988; Karhunen *et al.* 1990). *Phlebia radiata* 79 produces laccase in addition to LiPs and MnPs. It readily produces 1–3 isoforms of LiP, 1–2 isoforms of MnP and laccase, which were characterized and studied under different culture conditions (Hatakka *et al.* 1987; Hatakka and Lundell 1989; Karhunen *et al.* 1990; Vares *et al.* 1995). Recently the genes encoding three LiPs, two MnPs (Hildén *et al.* 2005; 2006) and two laccases (Saloheimo *et al.* 1989; Mäkelä *et al.* 2006) of *P. radiata* 79 were also thorouhgly characterized.

The efficiency of this fungus at degrading lignin was demonstrated in studies with ^{14}C-(ring)-DHP (Hatakka *et al.* 1983; Vares *et al.* 1994) and various ^{14}C-(lignin)-labelled lignocelluloses (Hatakka *et al.* 1983; Hatakka and Uusi-Rauva 1983). *Phlebia radiata* L12-41, the strain isolated in Sweden, also efficiently degrades lignin and is known to be a highly lignin-selective fungus (Ander and Eriksson 1977). The production of ligninolytic peroxidases by other *P. radiata* strains has been much less studied. Fungi in the genus *Phlebia* Fries appear to be very promising in biopulping, e.g. *P. radiata* (Ander and Eriksson 1977), *P. brevispora*, *P. subserialis* (Bourdot & Galzin) Donk and *P. tremellosa* (Eriksson and Kirk 1985; Akhtar *et al.* 1997), and *P. ochraceofulva* (Vares *et al.* 1993) or in feed applications, e.g. *P. tremellosa* cultivated on aspen (Reid 1985; 1989; Blanchette and Reid 1986) and in bioremediation (Kondo *et al.* 1994). *Phlebia tremellosa* has been frequently studied for its potential applications and lignin degradation, but there are controversial results as to the production of oxidative enzymes by this fungus (Vares *et al.* 1994). In the study of Hakala *et al*. (2004) *P. tremellosa* was one of the most selective fungi. Fackler *et al*. (2006) found that it was among the most active fungi, when delignification by selective lignin-degrading fungi were examined by the FT-NIR technique.

1.2.3 Extracellular lignin-degrading enzymes

White-rot fungi secrete into their cultivation medium several extracellular enzymes that operate close to the hyphae (Table 1.2). These enzymes may be bound to the hyphal cell walls or held close in a mucilage sheath (Kirk and Farrell 1987; Hatakka 2001). Extracellular lignin-degrading enzymes, such as laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), lignin peroxidases (LiPs, EC 1.11.1.14), manganese peroxidases (MnPs, EC 1.11.1.13) and versatile peroxidases (VPs, EC 1.11.1.16) are secreted extracellularly, usually at the onset of secondary metabolism (Hatakka 1994; Camarero *et al.* 1999). LiPs, MnPs and VPs

Table 1.2 Extracellular ligninolytic and cellulolytic enzymes involved in the degradation of wood components (modified after Hatakka 2001).

Enzyme and abbreviation	Donor, substrate or mediator	Main effect or reaction
Lignin peroxidase, LiP	H_2O_2, veratryl alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase, MnP	H_2O_2, Mn(II), organic acids as chelators, thiols, unsaturated lipids	Mn(II) oxidized to Mn(III); further oxidation of phenolic compounds to phenoxyl radicals
Versatile peroxidase, VP (hybrid peroxidase)	H_2O_2, same or similar compounds as for LiP and MnP	Same effect on aromatic and phenolic compounds as LiP and MnP
Laccase	O_2, phenols, mediators. e.g., HBT, ABTS	Phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators
Glyoxal oxidase, GLOX	Glyoxal, methylglyoxal	Glyoxal oxidized to glyoxylic acid; H_2O_2 production
Aryl alcohol oxidase, AAO	Aromatic alcohols (anisyl, veratryl alcohol)	Aromatic alcohols oxidized to aldehydes; H_2O_2 production
Cellobiose:quinone 1-oxidoreductase, CBQ	Cellobiose	Reduction of *o*- and *p*-quinones
Other H_2O_2-producing enzymes	Many organic compounds	O_2 reduced to H_2O_2
Endoglucanase (EG), *β*-glucanase (EC 3.2.1.4)	Amorphous cellulose	*β*-1,4-glycosidic bonds are hydrolysed randomly to glucose and cellooligo-saccharides
Cellobiohydrolase (CBH), exoglucanase, Cel 7A and Cel 7B (EC 3.2.1.91)	Crystalline and amorphous cellulose	Cellulose is hydrolysed cellobiose (and glucose)
β-glucosidase, cellobiase (EC 3.2.1.21)	Cellobiose	Cellobiose and cellooligosaccharides are hydrolysed to glucose

are haem-containing glycoproteins requiring H_2O_2 as an oxidant (Hatakka 2001; Martínez 2002). Laccases are copper-containing phenol oxidases that utilize molecular oxygen as oxidant. The other extracellular enzymes, such as H_2O_2-generating glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO, EC 1.1.3.7), are involved in lignin degradation and also develop at the onset of secondary metabolism (Ander and Marzullo 1997).

Based on their main extracellular ligninolytic enzymes, white-rot fungi can be classified into three or four groups: LiP and MnP group (e.g. *Phanerochaete chrysosporium, Phlebia radiata*), MnP-laccase group (e.g. *Ceriporiopsis subvermispora* (Pilát) Gilb. & Ryvarden), LiP-laccase group (e.g. *Phlebia ochraceofulva* (Bourdot & Galzin) Donk*, Junghuhnia separabilima* (Poulzar) Ryvarden and a fourth, the laccase group (*Pycnoporus cinnabarinus*

(Jacg.) Fr.) (Hatakka 1994). The MnP-laccase-producing fungi are the most common group (Hatakka 2001).

1.2.3.1 Lignin peroxidase (LiP)

LiP (EC 1.11.1.14) was first discovered in cultures of *P. chrysosporium* (Tien and Kirk 1983; Glenn *et al.* 1983). LiPs are haem-containing glycoproteins. They catalyse one-electron oxidations of phenolic and nonphenolic substructures, generating phenoxy radicals and aryl cation radicals (Kirk and Farrell 1987; Eriksson *et al.* 1990; Higuchi 1990; Hatakka 2001; Martínez 2002). The molecular masses of LiPs vary between 35 and 48 kDa and isoelectric points (p*I*) between 3 and 4. LiPs contain one high-spin ferric haem per enzyme molecule and are capable of catalysing a wide range of one- and two-electron oxidations (Tien and Kirk 1988; Hofrichter 2002). Typical reactions catalysed by LiP include C_α-C_β cleavage, C_α-oxidation, alkyl-aryl cleavage, aromatic ring cleavage, demeth(ox)ylation, hydroxylation and polymerization (Tien 1987; Higuchi 1990). LiP most preferentially catalyses the cleavage of C_α-C_β bonding in the propyl side chain of the model compound (Kirk and Farrell 1987; Hatakka 2001). The reactions of LiP have been studied, using lignin model compounds and synthetic lignins (Lundell *et al.* 1993; Hatakka 2001).

1.2.3.2 Manganese peroxidase (MnP)

MnP (EC 1.11.1.13) plays an important role in the depolymerization of lignin, because MnP in the presence of suitable organic acids is even able to mineralize lignin and lignin model compounds to considerable amounts (Wariishi *et al.* 1989; Hofrichter *et al.* 1999; 2002; Kapich *et al.* 1999). Among white-rot and soil-colonizing litter-decomposing fungi, MnP is the most common lignin-modifying peroxidase (Hofrichter 2002). MnP is a glycosylated haem protein with molecular masses from 38 to 62.5 kDa, averaging 45 kDa. The p*I* of MnP is acidic (pH 3–4) (Hofrichter 2002). As many as 11 isoforms of MnP were expressed in *C. subvermispora*, which is the most actively studied selectively lignin-degrading fungus (Lobos *et al.* 1994; Urzua *et al.* 1995; Fackler *et al.* 2006).

One-electron oxidations of phenolic and nonphenolic compounds are dependent on free Mn ions and an H_2O_2 (Glenn *et al.* 1986; Hofrichter 2002). MnP oxidizes Mn(II) to Mn(III), which then oxidizes organic substrates or phenolic rings to phenoxy radicals and this leads to decomposition of compounds. Highly reactive Mn(III) is stabilized by forming complexes with organic acids, e.g. lactate, malonate or oxalate, which are produced by fungi, and this chelated Mn(III) complex acts as a low-MW diffusible redox mediator. This mediator attacks recalcitrant phenolic lignin structures, generating unstable free phenoxy radicals or aryl cation radical intermediates that tend to disintegrate spontaneously (Hofrichter 2002). MnP is capable of oxidizing and depolymerizing natural and synthetic lignins as well as entire lignocelluloses in cell-free systems (*in vitro*) (Hofrichter 2002). Fungal MnP is not able to oxidize more recalcitrant nonphenolic lignin substructures without co-oxidants, such as unsaturated fatty acids and their derivatives (e.g. linoleic acid + Tween 80) *in vitro* (Kapich *et al.* 1999; Hofrichter 2002). Studies with white-rot fungi have shown that MnPs are more common than LiP (Hatakka 1994; 2001; Vares and Hatakka 1997; Steffen *et al.* 2000; Hofrichter 2002; Martínez 2002).

1.2.3.3 Laccase

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) has been known as a fungal enzyme since 1896 (Bertrand 1896) and reviewed by Baldrian (2006). Laccase is a blue copper oxidase, that usually contains four copper atoms per molecule in its catalytic centre (Thurston 1994; Baldrian 2006). Laccase contains one cysteine and 10 histidine residues,

which are involved in the binding of the copper atoms (Thurston 1994). Laccase is a monomeric, dimeric or tetrameric glycoprotein with a molecular mass between 60 and 80 kDa and its p*I* is between pH 3 and 5 (Thurston 1994; Baldrian 2006). The molecular mass of a laccase of *Phlebia radiata* 79 is 64 kDa and p*I* is 3.5 (Vares *et al.* 1995).

Laccase catalyzes four one-electron oxidations of mostly phenolic compounds such as catechol, hydroquinone, 2,6-dimethoxyphenol and syringaldazine to phenoxyl radicals (Thurston 1994; Eggert *et al.* 1996). Laccase has the capacity to oxidize also nonphenolic compounds if the reaction mixture is supplemented with substrates such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) or 1-hydroxybenzotriazole (HBT) (Bourbonnais and Paice 1990; Li *et al.* 1999). Laccase can cleave C_α-C_β bonds in the side chains of syringylglycerol-β-guaiacyl ether (Wariishi *et al.* 1985; Higuchi 1990), β_{-1} lignin substructures (Kawai *et al.* 1986) and demethoxylate methoxyl groups (Kirk *et al.* 1978; Eriksson *et al.* 1990). Laccase can be used in textile decolorization, pulp bleaching, effluent toxification and in bioremediation. The enzyme is produced by many filamentous fungi at bioreactor scale and is induced e.g. by Cu(II), VA and Tween 80 (Rodríguez Couto and Toca-Herrera 2007). Extracellular laccases are present in most fungal species, but most typically in basidiomycetous white-rot fungi and related litter-composting fungi (Steffen *et al.* 2000; Baldrian 2006). Almost all species of white-rot fungi produce extracellular laccase, e.g. *C. subvermispora* and *Phlebia* spp. The occurrence of laccase in brown-rot fungi is unclear (Baldrian 2006).

1.2.4 Brown-rot fungi

Brown-rot fungi preferentially attack dead coniferous wood, timber and wooden structures, in which they cause destructive types of decay and rapid degradation of cellulose (Kuhad *et al.* 1997; Goodell 2003). Winandy and Morrell (1993) showed that hemicellulose degradation by *Gloeophyllum trabeum* (Pers.) Murrill and *Postia placenta* (Fr.) Cooke is closely associated with wood strength loss. Brown-rot fungi can partially decompose lignin by demethoxylating it, but preferentially they degrade celluloses and hemicelluloses without removing the surrounding lignin. There is little outward evidence of degradation in the early stages of brown-rot decay, although mass losses can be between 5 and 10% in brown-rotted wood (Goodell 2003). Brown-rot fungi must first penetrate hemicelluloses to access cellulose, because cellulose microfibrils are enveloped by hemicellulose. They remove hemicellulosic xylose and mannose before cellulosic glucose. Hemicellulose hydrolysis may provide fungal enzymes with substrates for H_2O_2 production, which is necessary in the attack on lignocellulose by brown-rot fungi (Green and Highley 1997). In advanced decay stages, wood darkens and turns brown, which indicates the presence of modified lignin in wood. The wood loses strength and in the late degradation stages brown-rotted wood is a typically cubical, shrunken and crumbly material that easily breaks down into brown powder (Kuhad *et al.* 1997; Goodell 2003). Brown-rot fungi remove substances from the wood cell wall, first from the S_2 layer, and they may also attack the S_1 layer. The primary wall and the middle lamella are very resistant, due to their high lignin content (Dix and Webster 1995; Kuhad *et al.* 1997).

Demethoxylation of methoxyl groups and aromatic hydroxylation increase the number of phenolic hydroxyl groups. The increase in aliphatic hydroxyl and carboxyl groups is also clear based on an increased oxygen content that makes lignin chemically more reactive (Kirk *et al.* 1975; Eriksson *et al.* 1990; Jin *et al.* 1990b). It is still unclear if laccases and peroxidases are produced by brown-rot fungi. Phenol oxidases were found in cultures of *G. trabeum* (Ander and Eriksson 1978) and laccase by a spot test (Ejechi *et al.* 1996). Dey

et al. (1994) claimed that *Polyporus osteiformis* produced LiP and MnP. The oxidation of syringaldazine − a reliable indication of the presence of laccase − was detected in the brown-rot cultures of *Coniophora puteana* (Schumac.) P. Karst. (Lee *et al.* 2004). Score *et al*. (1997) found peroxidase and laccase from *C. puteana* and possibly laccase and peroxidase from *Serpula lacrymans* (Wulfen) J. Schröt, using a plate test. D'Souza *et al*. (1996) found laccase gene-specific sequences in *G. trabeum* and low laccase-like activity in the cultures of *G. trabeum* and *Postia placenta, P. balsamea* (Peck) Jülich and *Wolfiporia cocos* (F.A. Wolf) Ryvarden & Gilb. (Table 1.3). However, there is no laccase-encoding gene in the genome of *P. placenta* (Martínez, A.T., oral communication at the 10th International Conference on Biotechnology in the Pulp and Paper Industry (10th ICBPPI), Madison, WI, USA, 2007). *Gloeophyllum trabeum* did not produce extracellular laccase, LiP or MnP in the studies of Szklarz *et al*. (1989) or in the test of Kamada *et al*. (2002).

Table 1.3 Ligninolytic enzymes in some brown-rot fungi

Fungus	Enzyme	Reference
Lenzites trabea (*G. trabeum*)	phenol oxidases	Ander and Eriksson (1978)
Polyporus osteiformis	LiP (VA oxidation), MnP (oxidation Mn(II) to Mn(III))	Dey *et al.* (1994)
Gloeophyllum trabeum (3 strains), *Postia placenta, Postia balsamea, Wolfiporia cocos*	laccase (with ABTS at 418 nm)	D'Souza *et al*. (1996)
Gloeophyllum sepiarium, *Gloeophyllum* sp.	laccase (by spot test)	Ejechi *et al.* (1996)
Coniophora puteana	laccase, peroxidase (with plate test on malt extract agar)	Score *et al*. (1997)
Serpula lacrymans	laccase (?), peroxidase (with plate test on malt extract agar)	Score *et al*. (1997)
Coniophora puteana (4 strains)	laccase (with syringadazine at 526 nm)	Lee *et al.* (2004)

Fungi causing brown-rot-type decay include *Gloeophyllum trabeum* (syn. *Lenzites trabea*) (Haider and Trojanowski 1980; Ander *et al.* 1984; 1988; Ritschkoff *et al.* 1992; 1994; Hyde and Wood 1997; Irbe *et al.* 2001), *Poria placenta* (syn. *Postia placenta*, *Poria monticola*) (Ander *et al.* 1984; 1988; Jin *et al.* 1990a; Kim *et al.* 1991; Ritschkoff *et al.* 1995; Micales 1997; Irbe *et al.* 2001; 2006), *Coniophora puteana* (Kirk and Adler 1970; Kirk *et al.* 1975; Hyde and Wood 1997; Irbe *et al.* 2006), *Fomitopsis pinicola* (Sw.) P. Karst. (syn. *Fomes pinicola*) (Ander *et al.* 1988), and *Tyromyces palustris* (Berk. & M.A. Curtis) Murrill (Kamada *et al.* 2002).

1.2.4.1 *Gloeophyllum trabeum*

Gloeophyllum trabeum (syn. *Lenzites trabea*) Pers. ex Fr. is the most studied brown-rot fungus. As a very active fungus among other species of this group, it shares many properties with white-rot fungi (Kirk and Adler 1970; Kirk 1975; Haider and Trojanowski 1980; Ander

et al. 1984; 1988; Jin *et al.* 1990a; Kerem *et al.* 1999; Jensen *et al.* 2001; Trojanowski 2001; Goodell 2003; Cohen *et al.* 2004). *Gloeophyllum trabeum* is thermotolerant and grows well at high temperatures from 25 °C to 46 °C. It can resist temperatures as high as 60-80 °C for several hours (Viitanen 1994). Demethoxylation is a major initial reaction by brown-rot fungi. For example, *G. trabeum* strongly decreases the methoxyl contents of lignin in wood, resulting in an increase in carboxyl and phenolic hydroxyl groups (Kirk and Adler 1970; Haider and Trojanowski 1980). Nonphenolic units were less extensively demethoxylated than phenolic units in the studies of Haider and Trojanowski (1980). They also found that brown-rot fungi had a higher capacity for releasing $^{14}CO_2$ from methoxyl groups than white-rot fungi. The release of $^{14}CO_2$ is dependent on the added carbohydrate source, and the available carbohydrate especially influences the release of $^{14}CO_2$ from the methoxyl groups. Cellulose or hemicellulose or increased doses of glucose, cellobiose or xylose showed a repressive effect (Haider and Trojanowski 1980).

Gloeophyllum trabeum uses the extracellular Fenton reaction ($Fe(II) + H_2O_2$) to generate hydroxyl radicals (·OH), powerful oxidants that are assumed to be involved in wood degradation by brown-rot fungi. *Gloeophyllum trabeum* produces low-MW compounds named Gt chelators that catalyse the reduction of ferric ion to ferrous ion for use in the Fenton reaction (Goodell *et al.* 1997; Goodell 2003). Enoki *et al*. (1997) isolated a redox reaction-catalysing substance that converts H_2O_2 to ·OH.

1.2.4.2 *Poria (Postia) placenta*

Poria (*Postia*) *placenta* Fr. (syn. *Poria monticola*) Murr. is the first brown-rot fungus of which the entire genome has been deciphered (Martínez, A.T., oral communication at 10th ICBPPI, Madison, WI, 2007). It is may be the second most studied brown-rot fungus after *G. trabeum*. *Poria placenta* removes hemicellulose faster than cellulose in studies with spruce and birch wood (Davis *et al.* 1994). In their studies, *P. placenta* also caused demethoxylation of spruce lignin and an increase in vanillin-like structures and α-carbonyl moieties. In decayed birch wood the cleavage of *β*-O-4 linkages in lignin was the most prominent reaction, rather than demethoxylation. *Poria placenta* as well as *G. trabeum* is an efficient degrader of wood as analysed by ^{13}C NMR (nuclear magnetic resonance) spectroscopy, which showed that *P. placenta* primarily removes hemicellulose and methoxyl groups of lignin (Irbe *et al.* 2001). Other studies showed that *P. placenta* degrades amorphous cellulose more readily than crystalline cellulose regions (Irbe *et al.* 2006). The remaining cellulose after decay showed an increase in crystallinity (Davis *et al.* 1994). *Poria placenta* preferentially degraded polysaccharides in wood and produces large amounts of ·OH according to the studies of Kaneko *et al*. (2005). The authors suggested that brown-rot fungi produce extracellular ·OH as part of their wood-degrading system.

1.3 Low-molecular weight compounds and radicals in the degradation of wood

The fungal hyphae invade the wood cells through cuts and wounds and spread through the lumens of the wood cells to adjacent cells. The fungi secrete enzymes that decompose the polysaccharides, and then they penetrate from cell to cell either via pits or by producing boreholes in the wood cell walls. Lignin is degraded at some distance from the hyphae progressively from the lumens toward the middle lamella (Kirk and Farrell 1987; Green and Highley 1997). Brown-rot fungi rapidly depolymerize hemicellulose and cellulose, and more products accumulate than they can use. White-rot fungi successively degrade cell wall

carbohydrates only to the extent that they utilize them in fungal metabolism (Cowling 1961; Green and Highley 1997). Wood-decaying fungi are able to use wood as their only natural substrate, using extracellular enzymes and low-MW redox mediators, especially during the inital steps of lignin degradation (Xu and Goodell 2001; Martínez 2002).

Lignin degradation is extracellular, oxidative (nonhydrolytic) and nonspecific (Kirk and Farrell 1987). Extracellular ligninolytic and H_2O_2-producing enzymes as well as VA are secreted extracellularly by *P. chrysosporium* at the onset of secondary metabolism (Lundquist and Kirk 1978; Faison *et al.* 1986; Khindaria *et al.* 1996). White-rot fungi have nonenzymatic processes similar to that proposed for brown-rot fungi, involving the action of low-MW oxidating agents. Enzymatic and nonenzymatic systems may initiate brown-rot (Daniel 2003; Goodell 2003; Messner *et al.* 2003). Brown-rot fungi do not have the same enzyme systems as white-rot fungi do, although they have similar degradative capabilities to some extent as white-rot fungi (Hatakka 2001). Low-MW compounds, radical formation and production of organic acids may be involved in wood degradation by fungi, but there are similarities and differences between brown-rot and white-rot fungi (Goodell *et al.* 1997).

Low-molecular weight compounds

Fungal enzymes such as cellulases and peroxidases are too large to penetrate cell walls in sound wood, due to the limited size of the wood pores (Srebotnik and Messner 1991). The initiators of both cellulose and lignin breakdown may be small-MW compounds that can readily diffuse from the hyphae and penetrate into the wood cell and initiate decay (Evans *et al.* 1994; Wood 1994; Goodell *et al.* 1997; Shimada *et al.* 1997). The low-MW substances may be glycopeptides, phenolates or other types of iron-chelating compounds, e.g. siderophores, oxalate and simple aromatic compounds (Koenigs 1974; Fekete *et al.* 1989; Espejo and Agosin 1991; Jellison *et al.* 1991; Enoki *et al.* 1997; Goodell *et al.* 1997; Shimada *et al.* 1997). Many of these low-MW agents were isolated from cultures of both brown-rot and white-rot fungi (Hatakka 2001).

Several studies have shown that brown-rot fungi produce extracellular H_2O_2 in the wood cell walls in the early stages of decay, which may play an important role in the generation of ·OH in reaction with either a metal or a metal chelator (Enoki *et al.* 1997; Green and Highley 1997; Kim *et al.* 2002). The low-MW substances probably localize in the peripheral regions of the hyphal sheaths or extracellular slime that covers the hyphae of the fungus in the lumen. The fungal hyphae are thus in associatied with the extracellular glucan polymer-containing slime materials in the wood cell wall (Enoki *et al.* 1997). The slime produced by rot fungi probably plays an important role in wood decay processes, where it can form a microenvironment for lignin degradation reactions. Diffusion of enzymes through the slime layer into the cell lumen was confirmed by immunocytochemical examinations (Daniel 1994; Blanchette *et al.* 1997; Green and Highley 1997; Kim *et al.* 2002; Messner *et al.* 2003).

Fenton reaction

The wood decay fungi use an extracellular Fenton system ($Fe(II) + H_2O_2 \rightarrow Fe(III) + \cdot OH + OH^-$) to generate ·OH, a powerful oxidant that degrades wood (Koenigs 1974; Goodell *et al.* 1997; Hyde and Wood 1997; Kerem *et al.* 1999). These ·OH radicals are produced by extracellular reduction of Fe (III) and O_2, possibly by cellobiose dehydrogenase (CDH) or by nonenzymatically secreted hydroquinones (Ander and Marzullo 1997; Hammel *et al.* 2002). Extracellular glucose oxidase produced by brown-rot fungi can also be involved in degradation of wood (Green and Highley 1997). The decay fungi, e.g. *G. trabeum* and

P. chrysosporium, reduce an extracellular quinone to its hydroquinone, which then reacts nonenzymatically with Fe(III) to give Fe(II) and a semiquinone radical. The semiquinone reduces O_2 to give ·OOH and the original quinone (Hammel *et al.* 2002). Quinone redox cycling is known to be a source of ·OH, and it could provide an extracellular Fenton reaction in wood decay fungi. Several studies with *G. trabeum* have shown that it drives the Fenton reaction by continuously reducing extracellular benzoquinones to extracellular hydroquinone (mediated by extracellular quinone reductase), thus assuring a steady supply of electrons for the reduction of Fe(III) to Fe(II) and O_2 to H_2O_2 (Varela *et al.* 2003; Cohen *et al.* 2004; Suzuki *et al.* 2006).

Oxalic acid

Oxalic acid is produced by many white-rot and brown-rot fungal cultures and is one of the strongest organic acids (Green and Highley 1997; Micales 1997). Oxalic acid is secreted extracellularly at the onset of secondary metabolism. Brown-rot fungi accumulate oxalate during growth, but not white-rot fungi due to the presence of oxalate decarboxylase (ODC, EC 4.1.1.2), which degrades oxalate to formic acid and CO_2 (Green and Highley 1997; Micales 1997; Galkin *et al.* 1998; Mäkelä *et al.* 2002; Kaneko *et al.* 2005). Among the brown-rot fungi there are two exceptions, *G. trabeum* and *P. placenta*, which produce ODC enzyme, especially at low pH (Micales 1995; 1997). As the predominant chelator, oxalate binds Fe(III) in wood undergoing rot e.g. by *G. trabeum* and *Coniophora puteana* (Hyde and Wood 1997). Oxalate also plays an important role in white-rot fungi as a chelator of unstable Mn(III), which also affects the interactions of manganese ions at the active site of the MnP enzyme (Martínez 2002). The concentration of oxalic acid around the fungal hyphae appears to be tightly regulated and essential to in the decay process caused by brown-rot fungi (Micales 1997). Oxalate lowers the pH of the decay microenvironment and generates a pH gradient from the region outside the fungal cell wall and sequesters iron from cellulose (Goodell *et al.* 1997; Hyde and Wood 1997). ODC probably prevents the overaccumulation of oxalic acid, forming a buffered low-pH environment for the decay process (Goodell *et al.* 1997).

1.4 Analysis of fungal growth and activity

The growth requirements for fungal culturing and biodegradation assays based on ^{14}C-lignins were investigated mostly in the 1970s and 1980s, but this knowledge is still relevant today and has not advanced much since then. The studies then focused on the reaction mechanisms and crystal structures of lignin-degrading enzymes and on the analysis of degradation of lignocellulose by electron microscopy. Since the 1990s extensive research has focused on sequencing of genomes and genes that encode enzymes. In review articles (Kirk and Farrell 1987; Eriksson *et al.* 1990; Hatakka 2001), the authors listed important findings on the physiology of lignin degradation by *P. chrysosporium* and those which have then been found in studies of other fungi. These reviews showed that lignin degradation is (i) secondary metabolism and (ii) ligninolytic fungal cultures must include alternate carbon sources, such as hemicellulose, cellulose or added carbohydrates, (iii) low nutrient nitrogen (LN) contents, (iv) high oxygen tension or good aeration and (v) growth as mycelial mats rather than as submerged pellets in agitated cultures. These prerequisites were also found in *Phlebia radiata*, *P. tremellosa* and in many other white-rot fungi (Hatakka 2001). However, fungi can also degrade lignin under an air atmosphere and agitated culture conditions with detergent (Kirk and Farrell 1987).

1.4.1 Enzyme production

Phanerochaete chrysosporium or *Sporotrichum pulverulentum* Novobr., which is an anamorph of *P. chrysosporium*, was used as the main experimental fungus, and it typically produces LiPs and MnPs but atypically not laccase (Hatakka 2001). Lignin degradation starts by nitrogen starvation, when primary growth is turned to the secondary metabolic stage and the ligninolytic enzyme system of the fungus is synthesized. Secondary metabolism can also be triggered by carbohydrate or sulphur limitation (Kirk *et al.* 1978; Faison and Kirk 1985; Eriksson *et al.* 1990). Ligninolytic activity, VA production and peroxidase activity occurred at the same time on day 4 with *P. chrysosporium*, a phenomenon also observed in many other fungi (Eriksson *et al.* 1990). *Phanerochaete chrysosporium* and many other white-rot fungi metabolize carbohydrates and lignin in wood, but they need an alternative carbon source, such as cellulose, hemicellulose or added carbohydrate (Kirk and Farrell 1987).

White-rot fungi produce extracellular ligninolytic enzymes: laccases, LiPs and MnPs that are involved in lignin degradation. The occurrence of enzyme activity can vary between enzymes and fungi, but the separation methods used may also make interpretation of the results difficult (Hatakka 2001). The main LiP isoenzyme of *P. chrysosporium* is H8, when the fungus is cultivated under an oxygen atmosphere in nitrogen-limited or carbon-limited medium supplemented with VA (Farrell *et al.* 1989). The maximum specific activity of laccase observed in the primary metabolic stage (tropophase) in *Coriolus versicolor* (L.) Quél., while in *P. chrysosporium* cultures the maximum specific MnP activity was observed in the secondary metabolism phase (idiophase) (Szklarz *et al.* 1989). In the *P. chrysosporium* medium the wood mass loss was accompanied by an increase in protein production, and *Phellinus igniarius* (L.) Quél., another white-rot fungus, the protein secretion paralleled biomass production in the studies or Szklarz *et al.* (1989). Fackler *et al.* (2006) found no correlation between peroxidase activity and decrease in lignin.

The level of understanding of how isoenzymes are produced and used by fungi when they grow on natural lignocellulosic substrates, e.g. wood or straw, is still incomplete (Hatakka 2001). When the fungus grows in a complex medium such as wood chips, the overall patterns of LiP transcripts are different from those seen in defined media. Multiple forms of LiPs, MnPs, GLOX and laccase were detected and separated from culture extracts when *P. radiata* was grown on a solid lignocellulosic substrate, straw (Vares *et al.* 1995), but separation of MnPs and laccases from coloured straw extracts was difficult. The patterns of the enzymes were different from those usually found in liquid cultures of *P. radiata*. The presence of gene in a fungus is a convincing proof for the presence of enzyme (Janse *et al.* 1998; Hatakka 2001).

Veratryl alchol (VA)

VA (3,4-dimethoxybenzyl alcohol) is a natural inducer in several white-rot fungi, where it increases the initial rate of lignin degradation (Harvey *et al.* 1986; Valli *et al.* 1990). VA is also secreted by fungi and is a secondary metabolite and substrate for LiP (Conesa *et al.* 2002). VA was first found in cultures of *P. chrysosporium* and *Phlebia radiata* and then in many other white-rot fungi, e.g. *Sporotrichum pulverulentum*, *Pycnoporus cinnabarinus, Coriolus versicolor* and *Phlebia* (*Merulius*) *tremellosa* (Eriksson *et al.* 1990; Hatakka *et al.* 1991; 2001). *Phanerochaete chrysosporium* produces up to 0.2 mM VA as a secondary metabolite from glucose in LN cultures (Lundquist and Kirk 1978). LiP activity increased 2–4 h after supplementation of VA in cultures of *P. chrysosporium* (Faison *et al.* 1986) and five-fold in the studies of Kirk *et al*. (1986). LiP catalyses H_2O_2-dependent oxidation of

VA to veratraldehyde, forming the cation radical LiP II-VA•+ complex (Khindaria *et al.* 1996), and VA contributes to LiP oxidation of nonphenolic aromatic compounds (Martínez 2002). VA is used as the substrate in the LiP assay at 310 nm (Hatakka 2001). Harvey *et al.* (1986) suggested that LiP oxidizes substrates indirectly through the use of VA, which could act as a mediator between the substrates and enzyme. Valli *et al*. (1990) suggested that VA stimulates the oxidation of monomethoxylated aromatic compounds and protects LiP from inactivation of H_2O_2 rather than by acting as a mediator.

1.4.2 Radiorespirometric methods based on ^{14}C-labelled compounds

1.4.2.1 Lignin model compounds

Decomposition of synthetic ^{14}C-lignins to $^{14}CO_2$ for lignins synthesized with ^{14}C in the ring, side chain, or methoxyl group is considered a sensitive and definitive assay (Kirk *et al.* 1975; 1978). Radioactive lignins are prepared by specifically labelling lignin in plants or by *in vitro* synthesis (Kirk and Brunow 1988). Degradation of DHP and lignin model compounds can be used in measurements of the entire lignin-degrading capability of the fungus and also as a measure of ligninolytic enzymes because these enzymes are responsible for lignin depolymerization. Dimeric veratrylglycerol-*β*-guaiacyl ether (Fig. 1, in II), which has the most common intermonomeric *β*-O-4 ether linkage found in lignin, and monomeric veratric acid and vanillic acid (Fig. 1, in II) are the most often used models. It is believed that the same enzyme reactions that occur in the lignin model substrates also occur in lignin in the environment. The results show that fungi can degrade these compounds, including the opening of the benzene ring (Buswell and Odier 1987; Eriksson *et al.* 1990; Hatakka 2001).

1.4.2.2 Mineralization of radiolabelled lignins by fungi

Radiorespirometric methods have been used since the 1970s and are still considered the most reliable method for assaying lignin degradation. Lignin-related dimers (e.g. veratrylglycerol-*β*-guaiacyl ether), ^{14}C-(ring)- labelled DHP (dehydropolymerizate of coniferyl alcohol) or sometimes ^{14}C-labelled lignin monomer (e.g. vanillic acid) can be used as a model of lignin to reveal the ability of fungus to degrade lignin. In radiorespirometric systems the final degradation product, $^{14}CO_2$ released by the fungus, is collected.

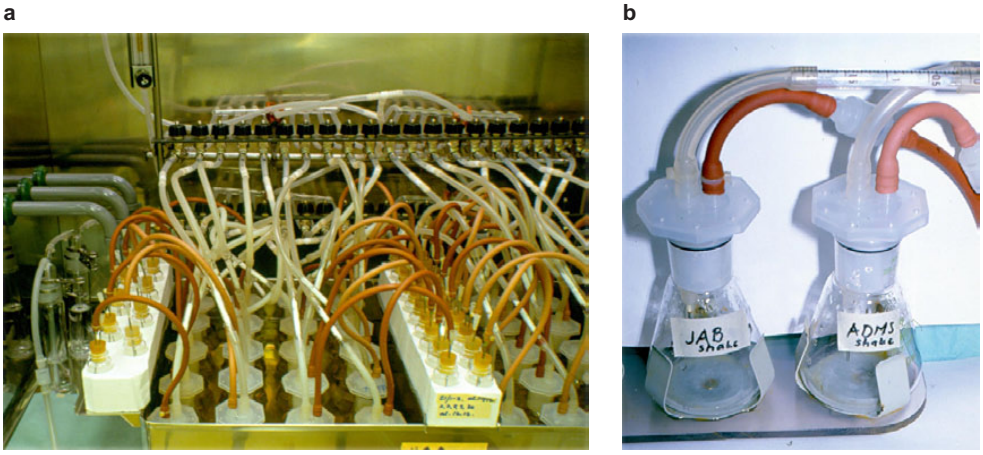


Fig. 1.4 a, b Radiorespirometric cultures in a water bath (a) and culture flasks fitted with stopper equipped with inlet and outlet port (b).

Development of $^{14}CO_2$ is highest during certain periods of growth, what is important to know. This time period differs between fungi and is also dependent on culture conditions (medium composition, temperature, atmosphere, aeration etc.).

Evolution of $^{14}CO_2$ from ^{14}C-labelled natural or synthetic lignin was determined for a variety of white- or brown-rot fungi (Kirk *et al.* 1975; Hatakka and Uusi-Rauva 1983; Crawford and Crawford 1988; Jin *et al.* 1990a; Vares *et al.* 1994; Hofrichter *et al.* 1999; Steffen *et al.* 2000; Tuomela *et al.* 2002) and preparations from different laboratories have given comparable results (Hatakka 2001). In these studies ^{14}C-labelled lignin or lignin model compounds are mineralized during secondary metabolism of the fungus to $^{14}CO_2$ and water, or water-soluble lignin fragments under aerobic culture conditions (Haider and Trojanowski 1975; Kirk *et al.* 1975; Hatakka and Uusi-Rauva 1983; Hatakka 2001). Usually only the end product, $^{14}CO_2$, is measured during cultivation. The conditions in the radiorespirometric system are optimal for lignin degradation, due to high oxygen tension, although fungi do not necessarily grow well under high oxygen tension (Vares *et al.* 1994; Hatakka 2001).

For radiorespirometry the fungal cultures are supplemented with the ^{14}C-labelled compound in a cultivation flask fitted tightly with a stopper equipped with inlet and outlet ports through which sterile oxygen or air can pass for continuous or periodic aeration for $^{14}CO_2$ collection purposes (Fig. 1.4 a, b). The growing fungus releases $^{14}CO_2$, which is led directly into an aqueous basic trapping solution containing a sample vial. After collection of $^{14}CO_2$ a scintillation cocktail is added and the radioactivity of $^{14}CO_2$ is quantified in a liquid scintillation counter. The accumulated $^{14}CO_2$ is presented as a percentage of the applied radioactivity or formation of $^{14}CO_2$ as a percentage per day of the applied ^{14}C (Hatakka *et al.* 1983). Samples are taken every day at the beginning of cultivation and every second or third day in older cultures. After incubation the solid residual is separated from the water-soluble lignin fragments by filtering and its radioactivity is analysed as well as ^{14}C from the solid residue by combusting to $^{14}CO_2$ and the recovery percentage from applied activity calculated. The total recovery of ^{14}C-label should be near 100%. The basic method is described in Hatakka and Uusi-Rauva (1983).

1.4.3 Biomass quantification by ergosterol analysis

Ergosterol (ergosta-5,7,22, trien-3β-ol) is the predominant sterol found in most mycelia-forming fungi, including the basidiomycota (Fig. 1.5 a). Ergosterol, like cholesterol in animals, occurs as a component of the phospholipid bilayer in the plasma membrane as free alcohols and also as esters or glycosides (Weete 1989; Gessner and Newell 2002). Fungi can synthesize different types of sterols. The proportion of ergosterol, e.g. in ascomycetes,

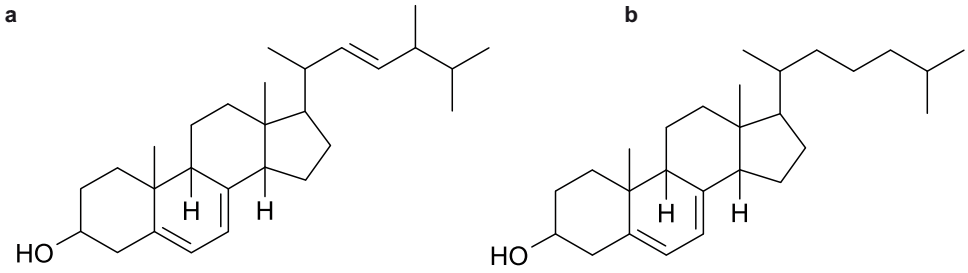


Fig. 1.5 a, b. Ergosterol (ergosta-5,7,22, trien-3β-ol) (a) and 7-dehydrocholesterol (b).

is from 79% to 100% of sterols and in basidiomycota from 40% to 85% (Lösel 1988; Weete 1989). Cultivated mushrooms, such as *Pleurotus ostreatus, Agaricus bisporus* (J.E. Lange) Pilát and *Lentinus edodes* (Berk.) Singer, have ergosterol in amounts of 83 – 89% of total sterols (Mattila *et al.* 2002). Little information is available on the ergosterol contents of basidiomycetous fungi. Since ergosterol is the predominant sterol component of most fungi and is produced only by fungi, it has been used as an indicator of fungal biomass (Seitz *et al.* 1977; Ruzicka *et al.* 2000; Gessner and Newell 2002). 7-Dehydrocholesterol (Fig. 1.5 b), of close structural similarity, often has been used as an internal standard.

Ergosterol is considered a sensitive and reliable marker of living fungal biomass, since its amount decreases in dead cells. It is labile on exposure to sunlight, ultraviolet (UV) radiation, heat and chemical reagents (Grant and West 1986; Newell *et al.* 1988; Gessner and Newell 2002). However, the studies of Gessner *et al*. (1991) and Mille-Lindblom *et al*. (2004) show that ergosterol can be rather stable as long as samples are protected from UV-radiation. Newell *et al*. (1988) showed that handling of samples, e.g. homogenization and refluxing, did not change the yield of ergosterol. In their studies the content of extracted ergosterol in samples kept at room temperature in the dark did not decrease at least for several days.

The use of the ergosterol assay has been applied mostly to mycorhiza in soil, plant material, leaf litter, food products and cereal grains (Gessner and Newell 2002). Initially ergosterol was used to indicate fungal contaminants in grain and cereals (Seitz *et al.* 1977; 1979) and then fungal biomass and mycorhiza in soil (Grant and West 1986; Salmanowicz and Nylund 1988; Montgomery *et al.* 2000; Ruzicka *et al.* 2000) and in forest litter, sediments and plant materials (Newell *et al.* 1988; Gessner *et al.* 1991; Barajas-Aceves *et al.* 2002; Mille-Lindblom *et al.* 2004). For decayed wood, ergosterol methods were applied by Nilsson and Bjurman (1990), Bjurman (1994) and Gao *et al.* (1993). Ergosterol measurement is commonly used as a marker for fungal biomasses in contaminated indoor environments and building materials (Saraf *et al.* 1997; Miller *et al.* 1998; Pasanen *et al.* 1999; Eikenes *et al.* 2005) and in foodstuffs, such as corn (Miller *et al.* 1983; de Castro *et al.* 2002), soybean (Dhingra *et al.* 1998) and in edible fungi (Mattila *et al.* 2002).

The ergosterol content of fungal mycelia varies widely, depending on species, growth stage, age and culture conditions, such as concentration of chemical composition in media, carbon source, C/N and C/P ratios, pH and temperature (Weete 1989; Tunlid and White 1992; Barajas-Aceves *et al.* 2002; Gessner and Newell 2002). There is probably little variation in ergosterol concentration in young fungal cultures from 2 days to 3 weeks (Seitz *et al.* 1979; Newell *et al.* 1988; Salmanowicz and Nylund 1988). Weete (1989) suggested that the amount of sterols increases cumulatively in cells during culture development. With *Rhizopus arrhizus* the amount of sterols and total lipids in the mycelia increases until midway between inoculation and onset of the stationary phase during a 6-days culturing time (Weete *et al.* 1973). Later, the sterol content decreases and then increases rapidly again. Weete *et al.* (1973) and Weete (1989) suggested that sterols are precursors to steroid hormones, and that that have stimulatory effects on the reproductive activities of certain fungi.

Ergosterol biosynthesis is probably dependent on the availability of oxygen. Messner *et al*. (1998) studied *Ceriporiopsis subvermispora* in different liquid media and bioreactor cultures with wood chips. The ergosterol content increased in these *C. subvermispora* cultures after a plateau stage at 14 days. Messner *et al*. (1998) noted that lower ergosterol concentrations coincided at the same time with intensive production of aerial mycelia growth. They suggested that the oxygen tension outside the wood chip was higher and

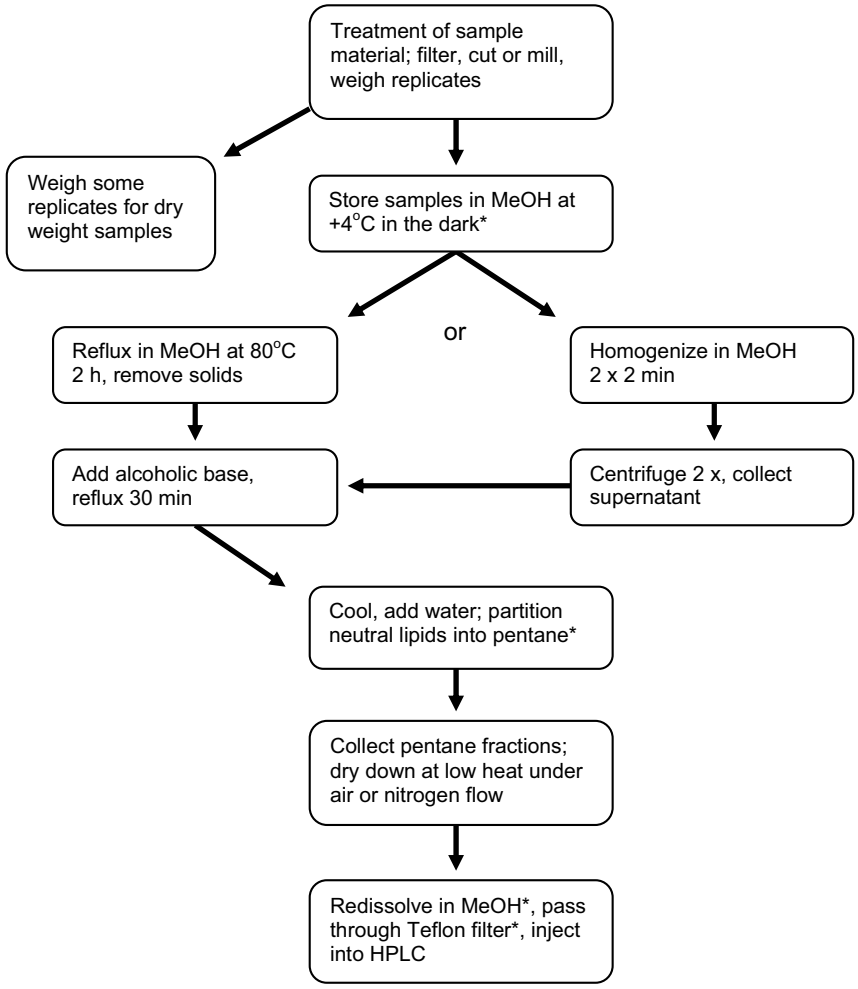


Fig. 1.6 Flow diagram of the basic steps of a suggested procedure for analysis of ergosterol for HPLC. The procedure is derived from Newell *et al*. (1988). *Points at which samples can be stored without loss of ergosterol. MeOH, methanol.

allowed intensive fungal growth. On the other hand, Gessner and Chauvet (1993) found that the ergosterol concentration was little affected by culture age with aquatic hyphomycetes. Their culturing time was 28 days and samplings were taken two or three times during that period.

The assay for ergosterol consists originally of extraction with methanol (MeOH), alkali saponification, which releases the bound sterol esters into free form, reextraction with the nonpolar solvents hexane or pentane, and determination by high-performance liquid chromatography (HPLC) at 282 nm (Fig. 1.6) (Seitz *et al.* 1977; Grant and West 1986; Newell *et al.* 1988). The sample treatment includes many steps, and the basic method is modified in many ways to shorten the sample treatment time (Table 1.4). The primary extraction step was omitted and the sample was subjected to direct saponification in the

method by Gessner *et al*. (1991). They found that 2 hours of refluxing before saponification in methanol and direct saponification were equally efficient at extracting ergosterol from fungal-colonized leaf litter samples. Ruzicka *et al*. (1995) omitted the saponification step and found no significant difference between the modified and standard methods. Seitz *et al*. (1977) employed ultrasonication and Young (1995) and Montgomery *et al*. (2000) a microwave-assisted extraction of the sample. Montgomery *et al*. (2000) found 2.5 – 9 times more ergosterol in soil samples with this method than with the routine method.

Dry weight (dw) is used as a measure for estimation of biomass as well as in basic calibration of other methods (Singh et al. 1994). The biomass of fungi in soil is traditionally determined by hyphal length measurements under the microscope (Gessner and Newell 2002). Fungal phospholipid fatty acid (PLFA) (fungi:18:2 ω 6,9) correlates with the amount of ergosterol and was proposed to be a good alternative indicator for ergosterol of living fungal biomass (Miller *et al.* 1998; Gessner and Newell 2002; Klamer and Bååth 2004). Adenosine triphosphate (ATP) occurs in all living organisms, but it has also been used as an indicator of fungal biomass (Gessner and Newell 2002). Suberkropp *et al*. (1993) compared ATP and ergosterol of fungal biomass associated with decomposing leaves in streams. They suggested that ergosterol is a more accurate indicator of fungal biomass than ATP. Chitin (a polymer of *N*-acetylglucosamine), is a major fungal cell wall component, but it is also present in bacterial cell walls and in the exoskeleton of invertebrates, and it was also derived from nonliving organic material (Nylund and Wallander 1992; Tunlid and White 1992; Singh *et al.* 1994). Ergosterol is easier and faster to analyze and it is accurate if compared to chitin analysing (Seitz *et al.* 1979; Suberkropp *et al.* 1993). Ergosterol appeared to be a more sensitive indicator of low levels of fungal mycelium than chitin or extracellular laccase enzyme in studies of *Agaricus bisporus* by Matcham *et al*. (1985). ELISA (enzyme-linked immunosorbent assay) and quantitative real-time PCR (polymerase chain reaction) are modern methods that have been used to determine microbial growth (Eikenes *et al.* 2005). DNA-based PCRs offer new possibilities for the detection of fungi in wood.

Lignin degradation in the environment occurs in solid wood or straw. It is necessary to be able to determine the amount of fungal biomass in solid substrate to know how the amount of fungal biomass and growth relates to the degradation efficiency. Therefore, here a commonly used method, the ergosterol assay was adopted to evaluate the mycelia growth. However, little information is available on basidiomycetous white-rot and brown-rot fungi. Separation experiments were carried out to determine the relationship of ergosterol biomass in the fungi during the lignin model compound degradation experiments.

Table 1.4 Preparation of samples for ergosterol analysis (HPLC, GC-MS or GC).

Sample description	Extraction	Hydrolysis	Extraction, partitioning and redissolving for analysis	Reference
1. SOIL				
Soil	Sonicated in methanol, filtered, washed with methanol, filtrate rotary evaporated	KOH and ethanol added to filtrate, refluxed for 30 min, H_2O added	Extracted 3x with hexane, extracts rotary evaporated, redissolved in methanol at 60°C, filtered, assays by HPLC	Grant and West 1986
Soil		Sample and methanol with KOH in ethanol homogenized and sonicated, in 85°C water bath for 30 min, H_2O added, filtered	Rinsed with methanol, extracted 3x with pentane, top layers pooled and dried at 60°C in evaporator under N_2. Dissolved in methanol, filtered and injected into HPLC	Eash *et al.* 1996 (simplified from Grant and West)
Soil or fungal mycelium		Sample in NaOH/methanol, in microwave oven for 17 and 18s, neutralized with HCl	Treated with methanol, extracted in pentane 3x, extracts filtered, evaporated under N_2 stream, methanol prior to HPLC or GC-MS analysis	Montgomery *et al.* 2000
Soil			Sample in hexane-propan-2-ol (98:2), methanol:ethanol (4:1), 20 ml hexane-propan-2-ol, ultrasonicated, top layer of hexane-propan-2-ol (spiked with ergosterol) transferred in tube, centrifuged, HPLC analysis on supernatant	Ruzicka *et al.* 1995, 2000
Fungal biomass and soil	Ultrasonificated in methanol for 3 min and centrifuged, washed 3x with methanol	In KOH/ethanol under reflux at 80°C for 1 h	Shaken vigorously with hexane, alcoholic phase washed twice with hexane, evaporated with rotary evaporator, rinsed 3x with methanol, same repeated, warmed and analysed with HPLC	Barajas-Aceves *et al.* 2002 (after Grant and West 1986)
2. LITTER				
Dead plant materials	Homogenized 2x in methanol, rinsed with methanol, centrifuged, repeated, or sample reflux in methanol for 2 h in water bath at 80°C	Sample added to KOH/ethanol, refluxed for 30 min, in H_2O	Neutral lipids into pentane, collected fractions dried down at 30°C under air or N_2, redissolved in methanol, filtered and injected into HPLC	Newell *et al.* 1988

Ground leaves	Mixed in methanol with Ultra Turrax, centrifuged, shaken (450 rpm for 5 min) with methanol, centrifuged	In KOH/ethanol refluxed for 30 min, filtered, in H_2O	Extracted with three 20-ml portions of petroleum ether, evaporated under stream of N_2, redissolved to dichloro-methane: isopropanol (99:1/v:v), centrifuged, injected into HPLC	Gessner *et al.* 1991
Dried litter, fungal mycelium (freeze-dried, homogenized)		In KOH/methanol with cyclohexane sonicated for 15 min, heated at 70°C for 90 min	In H_2O + cyclohexane, centrifuged, cyclohexane (upper) phase to new tube, washed with cyclohexane, combined phases evaporated under N_2 stream at 40°C, in methanol, sonicated, filtered, analysed with HPLC	Mille-Lindblom and Tranvik 2003 Mille-Lindblom *et al.* 2004 Klamer & Bååth 2004
3. MYCORRHIZA				
Mycorrhizal pine roots	Homogenized in ethanol, filtered	Refluxed with KOH (and pyrogallic acid as anti-oxidant) for 45 min, H_2O added, neutralized with HCl	Extracted 2x with n-hexane, both ethanol and hexane extracts evaporated and redissolved in ethanol. Prior to HPLC further purification with cartridge	Salmanowicz & Nylund 1988
4. FUNGAL SPORES, PELLETS, MYCELIUM				
Fungal cultures (grains)	Blended with methanol, mixture rinsed with methanol, centrifuged, same repeated.	Combined supernatants mixed with KOH/ethanol and refluxed for 30 min. Mixture diluted with H_2O.	Extracted twice with petroleum ether at 60-70°C. Extracts combined and evaporated to 10 ml with N_2, analysed with HPLC.	Seitz *et al.* 1977; 1979
Pellets from pine ecto-mycorrhizae	3x with ethanol, centrifuged	KOH/H_2O, extracted with hexane, evaporated	Redissolved in methanol, quantified on HPLC	Wallander *et al.* 1997
Fungal spores, mouldy bread or corn		Sample in methanol, treated with 0.5 ml of 2 M NaOH, microwave oven for 35 s, neutralized with HCl	Extracted 3x with pentane, evaporated, in methanol, analysed with HPLC and GC-MS	Young 1995
Fungal mass samples, (building material, dust)		In KOH/methanol, at 80°C for 90 min, H_2O added	Extracted twice with hexane, evaporated with N_2 stream, dissolved in dichloro-methane-hexane (1:1), TMS and pyridine added, TMS-derivatized samples dissolved in hexane, analysed with GC-MS	Saraf *et al.* 1997 Pasanen *et al*. 1999
Fungal mycelium or soil		NaOH/methanol, in microwave oven for 17 and 18 s, neutralized with HCl	Treated with methanol, extracted in pentane 3x, extracts filtered, evaporated under N_2 stream, methanol prior to HPLC or GC-MS analysis	Montgomery *et al*. 2000

Fungal mycelium and filter (lyofilized)		KOH and 2,6-di-tert-butyl-4-methylphenol (as antioxidant) in methanol-ethanol (3:2) refflux at 80°C for 30 min. Filtrated, rinsed 3x with methanol-ethanol, in H_2O	Partitioning with petroleum ether, concentrated, to HPLC vial, evaporated, washed 3x with petroleum ether under flow of N_2, in dichloromethane-isopropanol (100:1) for HPLC analysis	Charcosset & Chauvet 2001
Edible mushrooms (homogenized, freeze-dried)		In saturated aqueous KOH in ethanol at 85°C for 30 min, in H_2O	Extracted with cyclohexane, evaporated under N_2 stream, derivated to trimethylsilyl (TMS) ethers, analysed by GC-FID	Mattila *et al.* 2002
5. MEAL, CORN, GRAINS,				
Grains (fungal cultures)	Blended with methanol, mixture rinsed with methanol, centrifuged, same repeated.	Combined supernatants mixed with KOH/ethanol and refluxed for 30 min. Mixture diluted with H_2O.	Extracted twice with petroleum ether at 60-70°C. Extracts combined and evaporated to 10 ml with N_2, analysed with HPLC.	Seitz *et al.* 1977; 1979
Mouldy bread or corn, fungal spores		Sample in methanol, treated with 0.5 ml of 2 M NaOH, microwave oven for 35 s, neutralized with HCl	Extracted 3x with pentane, evaporated, in methanol, analysed with HPLC and GC-MS	Young 1995
Ground grains		Sample to ethanol/ H_2O with 10 ml 50% KOH, shaking gentle, reflux at 90°C for 30 min	Eluted in extraction column at least 15 min with n-hexane. Evaporated with rotary evaporator at max 40°C, dissolved in n-heptane for HPLC analysis	NMLK method no 172, 2002 (from Seitz *et al*. 1977; Zill *et al.* 1988; Schwadorf and Müller 1989)
6. BUILDING MATERIALS, DUST, GROUND WOOD				
Building material, dust, fungal mass samples		In KOH/methanol, at 80°C for 90 min, H_2O added	Extracted twice with hexane, evaporated with N_2 stream, dissolved in dichloro-methane-hexane (1:1), TMS and pyridine added, TMS-derivatized samples dissolved in hexane, analysed with GC-MS	Saraf *et al.* 1997 Pasanen *et al.* 1999
Ground wood samples			Extracted 12x in FBE unit with methanol, vacuum-evaporated, redissolved in 1 ml pyridine and derivated with 500 µl BSTFA for GC-FID	Eikenes *et al.* 2005

2 OBJECTIVES OF THE PRESENT STUDY

The general focus of this study was to determine how to monitor and measure the growth and activities of some wood-rotting basidiomycetous fungi. The potential ability of the fungi to produce lignin-degrading enzymes and demethylate lignin was also studied. Three methods were used (i) measurements of extracellular enzyme activities, (ii) radioisotopic methods by ^{14}C-labelled compounds and (iii) ergosterol analysis. Enzyme activity describes the biological activity of fungus, which is related to the fungal growth. The radioisotopic method, based on the production of $^{14}CO_2$ of a ^{14}C-labelled compound, is one of the most reliable methods for determining the growth and activities of fungi. However, the poor availability of radioactive isotope-labelled compounds, many of which are not commercially available, and absence of a well-equipped and -maintained isotope laboratory, could hinder the use of this otherwise excellent technique. Ergosterol is the predominant sterol in most fungi and is found almost exclusively in fungi. In contrast to many other compounds, it is a good indicator of biomass when it is necessary to follow the growth of fungi on a solid substrate.

Aims of the present study:

1 To determine the differences in fungi belonging to the genus *Phlebia* (and more specifically to the strains *P. radiata* L12-41 and *P. tremellosa* 2845, 76-24, 77-51 and 79-16) compared with the more widely studied *Phlebia radiata* 79 (ATCC 64658), as they relative to their enzyme activities and characteristics, and mineralization of ^{14}C-(ring)-labelled DHP (I),

2 To determine whether wood influences the demethoxylation of [O$^{14}CH_3$]-labelled lignin model compounds by fungi (II, III),

3 To determine the suitability of the ergosterol assay for indicating fungal growth on a solid substrate. (II-IV),

4 To determine how culture composition and time influence fungal growth on wood blocks and more specifically, whether dw correlates with ergosterol content and whether the latter correlates with the evolution of $^{14}CO_2$ or the production of enzymes by fungal mycelia(II-IV) and

5 To illustrate the advantages and disadvantages of the methods used here for analysis of the activities of wood-decaying fungi growing in wood. The main methods included measuring the lignin-degrading enzyme activities, the evolution of $^{14}CO_2$ from ^{14}C-labelled lignin or lignin model compounds and the biomasses as ergosterol contents that were related to fungal dw (I-IV).

3 MATERIALS AND METHODS

3.1 Methods used in publications I-IV

All fungal strains used in this work are listed in the Table 3.1. The strains are deposited at the culture collection of Fungal Biotechnology Culture Collection (FBCC) in the Department of Applied Chemistry and Microbiology at the University of Helsinki in Finland. The fungi were maintained on 2% (w/v) malt agar slants. The culture conditions are described elsewhere (I-IV).

Two DHP preparations, one from Dr. E. Odier and one from Prof. Kent Kirk, prepared of synthetic ^{14}C-(ring)-DHP, the nonphenolic lignin model compound [O^{14}CH$_3$]-labelled *β*-O-4 dimer at position 4 in the A ring, and [O^{14}CH$_3$]-labelled vanillic acid as well as nonlabelled DHPs and unlabelled β-O-4 dimeric compounds are described in the Table 3.2. Preparation of the ^{14}C-labelled materials for cultures is presented elsewhere (I-III). The setups of the experiments and methods are summarized in Table 3.3. More detailed descriptions are presented elsewhere (I-IV).

3.2 Other methods

3.2.1 Liquid flask cultures

Nonagitated liquid cultures

In nonagitated cultures *Phlebia radiata* L12-41 was grown in 100-ml conical flasks containing 10 ml of asparagine ammonium nitrate dimethylsuccinate LN-(ADMS) medium. ADMS medium is described in further detail elsewhere (III). Veratric acid was used as a stimulator and added 72 h after the inoculation to make a final concentration of 1.0 mM. Veratric (3,4-dimethoxybenzoic) acid (Fluka Chemie, Buchs, Switzerland) was dissolved in 0.1 M NaOH, adjusted with 0.1 M HCl to pH 5 and made up to the desired volume with deionized water. The solution was filter-sterilized.

Agitated flask cultures

Shake flask cultures of *Phlebia tremellosa* were grown in 100-ml conical flasks on a rotary shaker at 60 (Infors Ag C4 4103, 2.5-cm radius) or 180 rpm (New Brunswick, 5-cm radius; New Brunswick Scientific, Edison, NJ, USA) containing 25 ml LN-ADMS medium. VA (0.2 mM) and 0.05% (w/v) Tween 80 (sorbitan polyoxyethylene monooleate) (Fluka Chemie) were supplied in the medium when inoculated. VA alcohol (Fluka Chemie, Buchs, Switzerland) was distilled and dissolved in deionized water and filter-sterilized. Four replicates were used both for nonagitated and agitated cultures and incubated at 28 °C in the dark. The samples were collected every second or third day for analysis.

3.2.2 Separation of enzyme proteins

Ultrafiltration

The extracellular enzyme solutions from static liquid cultures of *P. radiata* (L12-41) and agitated cultures of *P. tremellosa* (2845) were pooled and filtered through a fibreglass filter (Whatman GF/A; Whatman, Maidstone, Kent, USA). The enzyme solution was concentrated to a final volume of 10 ml, using an Amicon ultrafiltration unit (YM membrane, 10-kDa cutoff; Amicon Inc., Beverly, MA, USA). The enzymes were then dialysed against 0.025 M sodium acetate buffer (pH 5.5).

Immunoblotting (Western blotting)

Immunoblotting was performed as described by Kantelinen *et al*. (1988). Antiserum against extracellular proteins of *Phlebia radiata* strain 79 (ATCC 64658) (Kantelinen *et al.* 1988) was kindly provided by Dr. Marja-Leena Niku-Paavola, VTT Biotechnology, Espoo, Finland.

Table 3.1 Fungal strains used in the experiments

Fungus	Origin	Used in
White-rot fungi		
Phanerochaete chrysosporium Burds., F1767 (ATCC 24725)	Obtained through M. Leisola, Eidgenössische Technische Hochschule (ETH), Zűrich, Switzerland (present address: Helsinki University of Technology, Espoo, Finland)	I, II, IV
Phlebia radiata Fr., 79 (ATCC 64658)	Isolated by A. Hatakka and T. Pirhonen, Department of Applied Chemistry and Microbiology, University of Finland, Helsinki, Finland	I, II, IV
Phlebia radiata Fr., L12-41	Obtained through K.-E. Eriksson, from T. Nilsson, Swedish University of Agricultural Sciences, Department of Forest Products, Uppsala, Sweden	Enzyme purif.
Phlebia tremellosa (Schrad.,Fr.) Nakas. & Burds. (= *Merulius tremellosus*) PRL 2845 (ATCC 48745)	Obtained from I. Reid, Pulp and Paper Research Institute of Canada (PAPRICAN)	Enzyme purif.
Phlebia tremellosa (Schrad.,Fr.) Nakas. *et*. Burds. (= *Merulius tremellosus*) 76-24, 77-51 and 79-16	Obtained through the courtesy of E. Parmasto, Institute of Zoology and Botany, Tartu, Estonia	I
Physisporinus rivulosus (Berk. & Curtis) Ryv. T241i (DSM 14618)	Isolated in the south of Finland. Department of Applied Chemistry and Microbiology, University of Finland, Helsinki, Finland. Hakala *et al*. (2004; 2005)	IV
Ceriporiopsis subvermispora (= *Gelatoporia subvermispora*) (Pilát) Gilbn. & Ryv., CZ-3-FPL (ATCC 96608)	Obtained from US Forest Products Laboratory, Madison, Wisconsin, USA	IV
Brown-rot fungi		
Gloeophyllum trabeum (= *Lenzites trabea*) Pers. ex Fr., strain 83	Obtained from A. Leonowicz, University of Maria Curie-Sklodowska, Lublin, Poland	III, IV
Poria (Postia) placenta Fr., FPRL 280	From VTT Biotechnology, Espoo, Finland	III, IV

Table 3.2 ^{14}C-labelled synthetic lignin and aromatic model compounds

Type	Specific activity	Source	Used in
^{14}C-ring-labelled DHP (dehydropolymerizate of coniferyl alcohol), with molecular mass 4 –10 kDa	277 kBq/mg	E. Odier (Eriksson *et al*. 1990) INRA, Grignon, France	I
^{14}C-ring-labelled DHP, molecular mass 1600 kDa	15 kBq/mg	From K. Kirk, US Forest Products Laboratory, Madison, Wisconsin, USA	Radio-isotope experiment
Guaiacyl-type DHP	Nonlabelled	G. Brunow, Laboratory of Organic Chemistry, University of Helsinki, Finland	I
[O^{14}CH$_3$]-4-hydroxy-3-methoxy-benzoic (vanillic) acid	13.7 MBq/mg	Obtained from J. Rogalski, University of Marie Curie Sklodowska, Lublin, Poland	II
[4-methoxy-^{14}C] veratryl-glycerol-*β*-guaiacyl ether (*β*-O-4 dimer)	645 kBq/mg	See Hatakka *et al*. (1991)	II, III
Veratrylglycerol-β-guaiacyl ether (*β*-O-4 dimer)	Nonlabelled	See Hatakka *et al*. (1991)	II, III

3.3 Statistical analysis

The results are expressed as means of four parallel cultures ± standard deviations. Significant differences between culture medium groups were identified, using one-way analysis of variance (ANOVA) in results of ^{14}C-labelled isotope cultures and ergosterol analysis. Tukey's test was applied after ANOVA to compare means at $P < 0.05$. All statistical analyses were performed with the program SPSS 10.0 for Windows (II-IV). Regression analysis was performed from results of liquid cultures between ergosterol contents and dw with the SigmaPlot 8.0 program (IV).

Table 3.3 Methods used in this study

Assay/Method	Described in
Cultivation of fungi	
- growing of fungal inocula	I-IV
- liquid bioreactor cultures (2 l) for enzyme assays and purification	I
- agitated liquid cultures (25 ml/100 ml) for enzyme assays and purification	I
- stationary liquid cultures (10 ml/100 ml) for enzyme assays and purification	I
- stationary liquid cultures (10 ml/100 ml) for ergosterol analysis	IV
- solid agar and wood block on agar ^{14}C-cultures and for ergosterol analysis (10 ml/100 ml)	II, III
- agitated ^{14}C-cultures (25 ml/100 ml)	Radioisotope experiment
- stationary liquid ^{14}C-cultures (10 ml/100 ml)	I
Determination of enzyme activity and protein	
- enzyme assays: LiP, MnP, laccase, GLOX	I
- extracellular protein (Bradford method)	I
Separation and characterization of extracellular proteins	
- fast protein liquid chromatography (FPLC)	I
- SDS-PAGE	I
- Western (immuno) blotting	Enzyme purification
- isoelectric focusing (IEF)	I
Radiorespirometric method	
- collecting of $^{14}CO_2$	I-III
- liquid scintillation counting	I-III
- combustion of ^{14}C-material	I
Ergosterol analysis	II-IV
High-performance liquid chromatography (HPLC)	II-IV

4 RESULTS AND DISCUSSION

4.1 Production of lignin-modifying enzymes of *Phlebia radiata* and *Phlebia tremellosa*

4.1.1 Culture conditions for the production of lignin-modifying enzymes

The fungal strains *Phlebia radiata* L12-41, isolated from Sweden, and *Phlebia tremellosa* 2845 (ATCC 48745), isolated from Canada, are known to be highly lignin-selective white-rot fungi (Ander and Eriksson 1977; Hatakka *et al.* 1983; Blanchette *et al.* 1985; Reid 1985; 1989) and were studied in the present investigation. The goal was to determine whether these fungi produce the same oxidative enzyme systems as characterized for the well-known *Phlebia radiata* 79 (ATCC 64658) isolated from Finland (Hatakka and Uusi-Rauva 1983; Karhunen *et al.* 1990; Niku-Paavola *et al.* 1990; Lundell and Hatakka 1994; Moilanen *et al.* 1996; Hildén *et al.* 2005; 2006; Mäkelä *et al.* 2006) and three *P. tremellosa* strains received from Estonia, previously characterized and here described (I).

Phlebia radiata L12-41 and *P. tremellosa* 2845 were cultivated under different conditions to enhance the production of lignin-modifying enzymes. Nonagitated flasks were induced with 1.0 mM veratric acid and shake flasks with 0.2 mM VA. In addition, *P. tremellosa* 2845 was cultivated at different agitation speeds.

4.1.2 Production of enzymes by *P. radiata*

Under static culture conditions the enzyme production patterns of *P. radiata* L12-41 resembled those of agitated and bioreactor cultures of *P. radiata* 79 (Hatakka *et al.* 1989; Kantelinen *et al.* 1989; Lundell *et al.* 1990; Moilanen *et al.* 1996). At first, both fungi produced laccase, which attained a sharp maximum on day 5 and later began to produce LiP and MnP (I).

4.1.3 Production of enzymes by *P. tremellosa*

Significant differences in mycelial growth were observed in agitated cultures of *P. tremellosa* 2845, that were induced by addition of 0.2 mM VA. In these cultures about one third of the flasks contained only a few large mycelial pellets, with an average diameter of 10–15 mm. This phenomenon was not found in *P. radiata*, although the inoculation and cultivation were performed in the same way. The highest LiP and MnP activities were obtained from *P. tremellosa* 2845 cultures with large mycelial pellets. The maximum production of enzymes occurred rather late, e.g. LiP reached 1100 nkat l^{-1} on days 17–21. The majority of flasks contained small pellets, and these cultures showed high laccase activities but production of LiP or MnP was very poor. Periodic shifts to increase the shaking rates from 60 rpm to 180 rpm resulted in actively LiP- and MnP-producing mycelia. However, high levels of LiP or MnP activities were not achieved in the flasks containing large mycelial pellets generated by continuous high rotation speed in this experiment.

The mycelial pellet size strongly correlated with the activities of LiP and MnP; the larger the mycelial pellets, the higher were the levels of LiP and MnP here produced by *P. tremellosa* 2845. Reid (1985) and Janshekar and Fiechter (1988) showed that pellet size may affect lignin degradation, since *P. chrysosporium* degrades lignin in agitated cultures, forming large pellets. In submerged cultures, the mycelial pellets may vary from loose flocculent to compact spherical (Carlile *et al.* 2001). In these cultures the large mycelial

pellets appeared to be loose and flocculent, and in the same flasks there were also small mycelial pellets. Jimenéz-Tobon *et al*. (1997) showed that the highest MnP activity was obtained with intermediate agitation rates (130–150 rpm) and the average final size of the mycelial pellets was much larger at the lower inoculation level. The authors suggested that the average final pellet size directly determines the efficiency of enzyme production. Leisola *et al*. (1985) and Žmak *et al*. (2006) showed that the time needed for the onset of LiP production by *P. chrysosporium* with large pellets was longer than that observed with small pellets. This was not found in the present study in *P. tremellosa* 2845; the time needed for the highest LiP activity was 13–15 days in the flasks with larger mycelial pellets, while in the flasks with small mycelial pellets, very low levels of LiP or MnP activity were detected. With *P. tremellosa* 2845, a relationship between the mycelial pellet size and the production of LiP and MnP apparently occurred, but the reason for this relationship is not clear.

4.1.4 Separation of enzymes from *P. radiata*

For the separation of lignin-modifying enzymes from *P. radiata* L12-41, cultures from the stationary flasks induced by addition of 1.0 mM veratric acid were harvested on day 14 when the highest LiP activity (800 nkat l^{-1}) was obtained. Three haemoproteins named h1, h2 and h3 were separated (absorbance at 405 nm) (Fig. 4.1a). Two of these peaks showed LiP activity (h1 and h3, Fig. 4.1b) and two exhibited MnP activity (h2 and h3, Fig. 4.1b). Peak h1 corresponded to LiP with a molecular mass of 39 kDa (LiP1, Table 4.1) and h2 MnP showed molecular mass of 47–48 kDa (MnP2) as analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4.3a). The p*I* values were 4.2 and

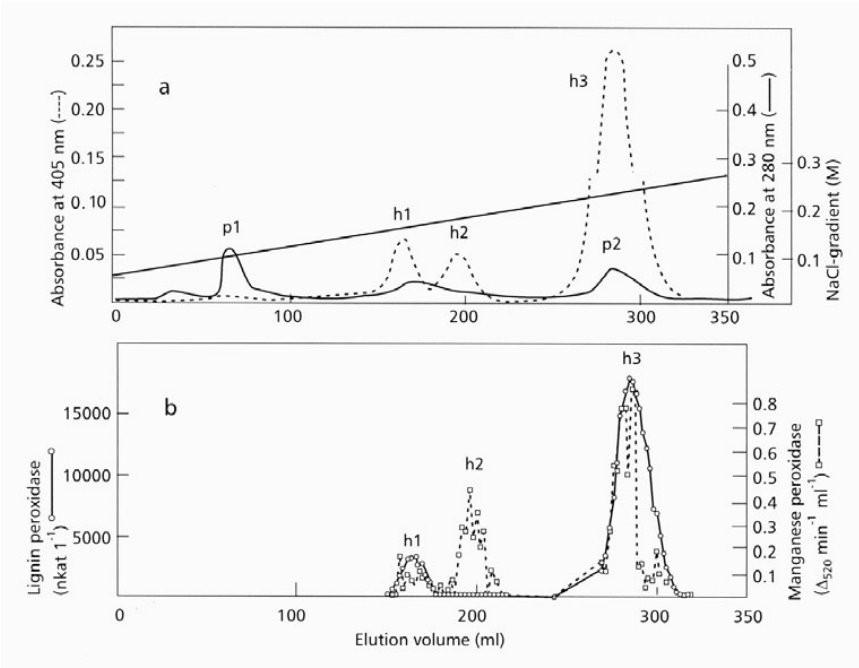


Fig. 4.1 FPLC elution profile of extracellular proteins from stationary 1.0 mM veratric acid-induced cultures of *Phlebia radiata* L12-41 harvested on day 14. Eluted protein fractions monitored at 280 (solid line) and haem 405 nm (broken line). P, protein peak; h, haemoprotein peak (a). h1, LiP; h2, MnP; h3, LiP and MnP (b).

3.75, respectively (Fig. 4.1 and Table 4.1). Peak h3 consisted of two proteins with molecular masses of 39–40 kDa (LiP2) and 44–45 kDa (MnP1) and p*I* values 3.1 and 3.7, respectively (Fig. 4.3a, Fig. 4.4a and Table 4.1); they could not be separated.

Table 4.1 Properties of the lignin-modifying enzymes of *Phlebia radiata* 79. *P. radiata* L12-41, and *P. tremellosa* 2845, 76-24, 77-51 and 79-16. Results of the three latter fungi are from article I.

Fungal strain	Enzyme	Molecular mass (kDa)	p*I*	Immunological reaction[a]
P. radiata 79[b]	LiP1[c]	40	4.1	+
	LiP2	45	3.9	+
	LiP3	44	3.2	+
	MnP1[d]	47	4.7	+
	MnP2	48	3.8	nd
	MnP3	47–50	3.6	nd
	Lac1	64	3.2-3.5	-
	GLOX	67	4.5	-
P. radiata	LiP1	39	4.2	+
L12-41	LiP2[e]	39–40	3.1	+
	MnP1[e]	44–45	3.7	+
	MnP2	47–48	3.75	+
	Ex[f]	70	nd	nd
P. tremellosa	LiP1	35–36	3.1	+
2845	LiP2	38–39	3.5	+
	LiP3	40	4.0	+
	MnP	nd	nd	nd
	Laccase	nd	nd	nd
	Ex[f]	68–70	nd	nd
P. tremellosa	LiP1 H1	45	3.75	nd
76-24	LiP H2	41	3.25	nd
	Laccase	nd	nd	nd
P. tremellosa	LiP H1	45	3.75	nd
77-51	LiP2	41	3.25	nd
	Laccase	67	3.25	nd
	GLOX	67	4.5	nd
P. tremellosa	LiP H1	45	3.75	nd
79-16	LiP H2	41	3.25	nd
	Laccase	nd	nd	nd

[a]Western blotting, reaction (+) or (-) with antiserum against *P. radiata* 79 LiP L1 provided by Dr. Marja-Leena Niku-Paavola (Kantelinen *et al.* 1988)

[b](Lundell 1993; Vares *et al.* 1995; Moilanen *et al.* 1996; Hildén *et al.* 2005)

[c]Lignin peroxidase

[d]Manganese peroxidase

[e]LiP2 and MnP1 were not separated

Ex[f] = protein (not haem)

nd = not determined

The lignin-modifying enzyme production profiles of the well-studied *P. radiata* 79 (Hatakka *et al.* 1987; 1989; Kantelinen *et al.* 1988; Niku-Paavola *et al.* 1988; 1990; Karhunen *et al.* 1990) and the here studied *P. radiata* L12-41 were similar. In liquid cultures, both fungi produced 2–3 isoforms of LiP, MnP and laccase. Molecular analysis of *P. radiata* 79 showed that it has at least three LiP-, two MnP- and two laccase-encoding genes (Hildén *et al.* 2005; 2006; Mäkelä *et al.* 2006).

In *P. radiata* L12-41, laccase activity appeared first and the LiPs and MnPs appeared and later achieved their maximum activities. The production kinetics with L12-41 slightly differed from those of *P. radiata* 79 laccase. The highest laccase activity of *P. radiata* L12-41 appeared on day 5, whereas with *P. radiata* 79 the highest level was obtained as early as on the third day (Kantelinen *et al.* 1989). *Phlebia radiata* L12-41 was much slower in the production of LiP and MnP; the highest activities were achieved during days 11–15, whereas with *P. radiata* 79 the highest activity was achieved on days 6–8 (Hatakka *et al.* 1989).

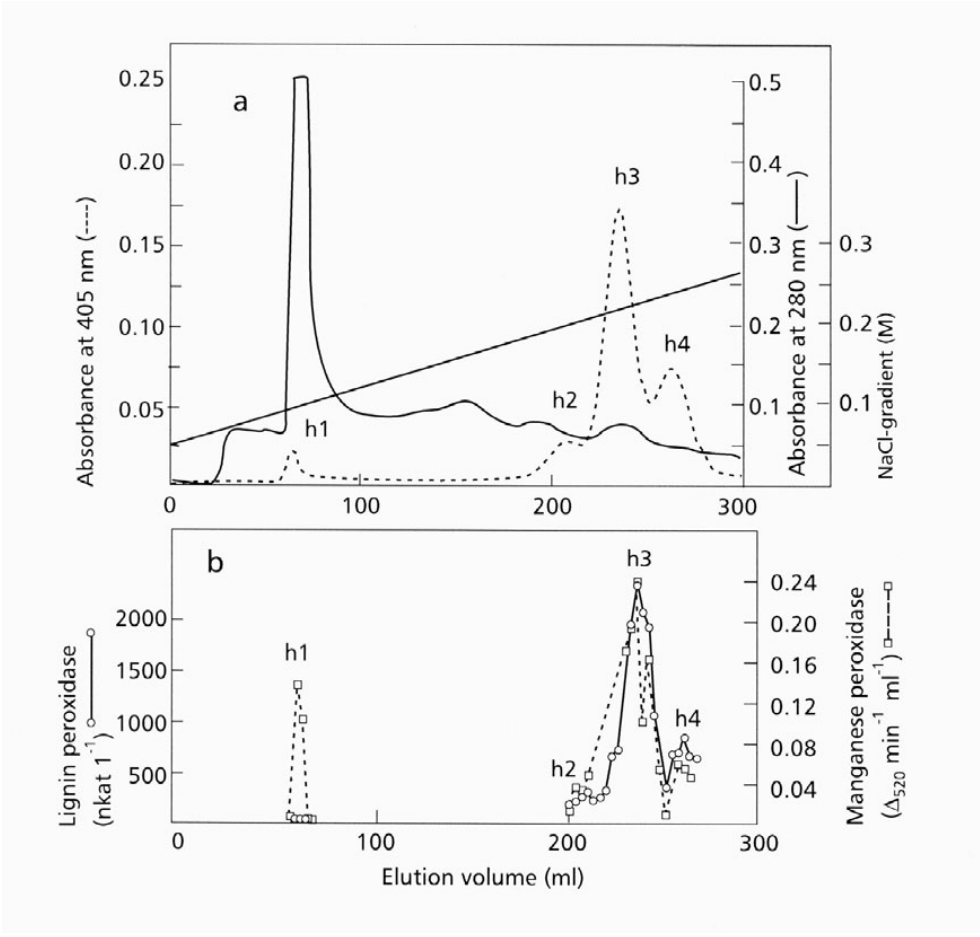


Fig. 4.2 FPLC elution profile of extracellular proteins from *Phlebia tremellosa* 2845 cultures with large pellets, harvested on days 18–21. Eluted protein fractions were monitored at 280 nm (solid line) and haem 405 (broken line) (a). Symbols: h2–h4, LiPs (b).

4.1.5 Separation of enzymes from *P. tremellosa*

Figure 4.2 shows separation of the extracellular enzymes from *P. tremellosa* 2845. The culture filtrates were harvested only from shake flasks supplemented with 0.2 mM VA and containing large mycelial pellets, as discussed previously. In these cultures 600 nkat l^{-1} of LiP activity was obtained. Four haemoproteins absorbing at 405 nm were detected. Three separate isoforms showing LiP activity (peaks h2–h4) were apparently present. Peaks h2, h3 and h4 corresponded to LiPs with molecular masses of 40 (LiP3), 38–39 (LiP2) and 35–36 (LiP1) kDa, as determined by SDS-PAGE (Fig. 4.3a). Their p*I* values were 4.0, 3.5 and 3.1, respectively (Fig. 4.4 and Table 4.1).

MnP was unstable in cultures of *P. tremellosa* 2845. The fungus produced MnP activity, which disappeared, and separation and purification of the enzyme were unsuccessful. Higher MnP activities were obtained with 10-fold manganese (Mn(II) ions) concentration in the bioreactor cultures but shake flask cultures with basal Mn(II) content were used for enzyme purification. A higher Mn(II) level could have stabilized the activity of the enzyme.

It is apparent that Mn(II) in the medium regulates the production of lignin-modifying peroxidases; addition of Mn(II) ions predominantly causes production of MnP and repression of LiP. Such clear regulation of lignin-modifying enzymes was reported by Rüttimann-Johnson *et al*. (1993) and Bonnarme and Jeffries (1990) with *Phlebia radiata, P. tremellosa* and *P. subserialis* with different Mn(II) concentrations. The latter authors found that high concentrations of Mn(II) had a promoting effect on MnP activity and a repressive effect on LiP activity. The authors observed production of MnP in *P. tremellosa* (strain 2845) under an oxygen atmosphere with low (0.32 ppm) and high (39.8 ppm) Mn(II) concentrations, but they detected no LiP activity in *P. tremellosa* or *P. subserialis*. It should be noted that the basic level of Mn(II) in their medium was 11.15 ppm, which is 100-fold more than in our medium, where the basal concentration of Mn(II) was 0.12 ppm (2.4 μM).

The influence of manganese in *P. radiata* 79 cultures with and without Mn(III)-chelating malonate was also studied by Moilanen *et al.* (1996), who found that high Mn(II) (180 μM, 9 ppm) together with malonate increased the levels of MnP and laccase production. However, high levels of Mn(II) alone had no influence on the production of enzymes, while cultures with high levels of Mn(II) were less effective in the mineralization of ^{14}C-labelled DHP. Bonnarme and Jeffries (1990) showed that in several fungi, e.g. *P. chrysosporium* and *Phlebia* spp., the rate of $^{14}CO_2$ release from ^{14}C-labelled DHP increases at low Mn(II) levels and decreases with high Mn(II) concentration, pointing to the importance of LiP for lignin mineralization.

4.1.6 Characterization of enzymes

The molecular masses and p*I* levels for separated lignin-modifying enzymes are summarized in Table 4.1. Extracellular proteins from *P. radiata* L12-41 and *P. tremellosa* 2845 showing peroxidase activity reacted with antiserum raised against *P. radiata* 79 LiP (Fig. 4.3b). Similar LiP isoenzymes were apparently expressed in all of the two *P. radiata* strains (79, L12-41) and *P. tremellosa* 2845 studied here (Table 4.1). However, LiP isoenzymes from the three *P. tremellosa* strains, (76-24, 77-51 and 79-16) were very similar.

Phlebia tremellosa 2845 and the three other *P. tremellosa* strains (I) also produced laccase and 2–3 isoforms of LiP, in accordance with the *P. radiata* strains. Separation of the extracellular proteins from the bioreactor cultures of *P. tremellosa* strains (76-24, 77-51 and 79-51) containing both low and high concentrations of Mn(II) and supplemented with

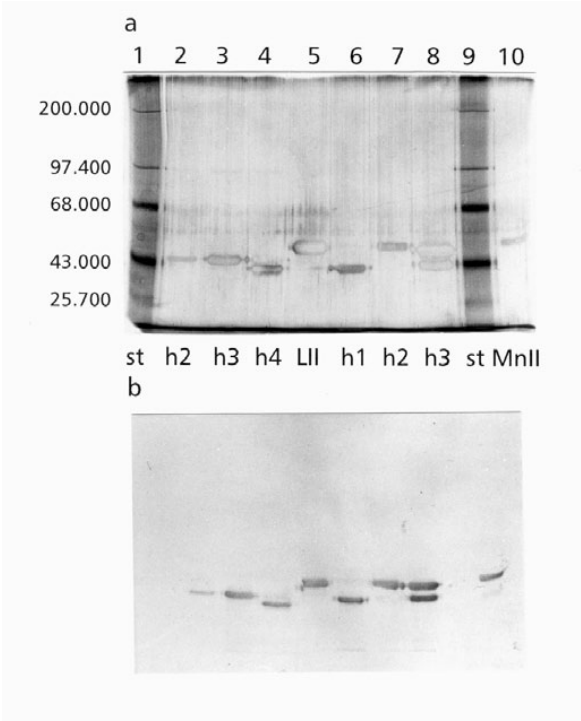


Fig. 4.3 SDS-PAGE (a) and Western blotting (b) of LiP and MnP of *Phlebia tremellosa* 2845 and *Phlebia radiata* L12-41. Molecular mass standards (lanes 1 and 9, indicated on the left in daltons, Da) and proteins of LiP2 and MnP of *P. radiata* strain 79 as standard (lanes 5 and 10, respectively).

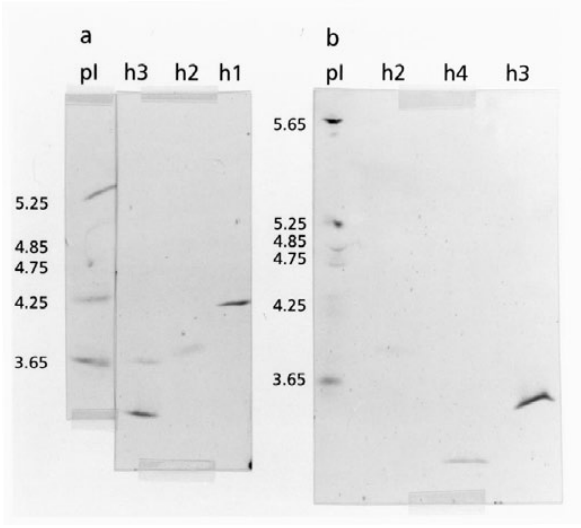


Fig. 4.4 Isoelectric focusing of ligninolytic enzymes purified from *Phlebia radiata* L12-41 (a) and *Phlebia tremellosa* 2845 (b). Haemoprotein peaks are shown as in Figs. 4.1 and 4.2. The p*I* values of standard proteins are indicated on the left.

malonate did not confirm production of MnP (I). *Phlebia tremellosa* 2845 produced low activities of LiP and MnP. Biswas-Hawkes *et al.* (1987) also had difficulties in showing any LiP activity in *P. tremellosa* (syn. *Merulius tremellosus*) FPRL13. Some studies indicate that the presence of detergent compounds, e.g. Tween 80, facilitates the detection of LiP (Jäger *et al.* 1985). On the other hand, the detergents are able to protect lignin-modifying enzymes of *Phanerochaete chrysosporium* against mechanical inactivation during agitation, as pointed out by Venkatadri and Irvine (1990). Addition of Tween 80 to shake flask cultures at the end of primary growth resulted in the production and maintenance of high levels of LiP activity for several days under conditions of high agitation (Venkatadri and Irvine 1990). With *P. tremellosa* 2845 the addition of Tween 80 had no influence on the stability of MnP in shake flasks in the present study.

The production patterns of LiP and laccase in *P. radiata* L12-41, *P. tremellosa* 2845 and the three other *P. tremellosa* strains (76-24, 77-51 and 79-16) resembled those reported for *P. radiata* 79 (Kantelinen *et al.* 1989; Lundell *et al.* 1990; Moilanen *et al.* 1996). In all of these fungi, laccase activity appeared early (days 1 – 5) and usually attained its maximum level rapidly before decreasing with simultaneuously increasing LiP activity. The Canadian strain *P. tremellosa* (PRL-2845) degrades lignin selectively from the cell walls of aspen and birch xylem (Blanchette and Reid 1986; Blanchette *et al.* 1987). The degree of genetic variation of these *Phlebia* strains were studied, using 18S rDNA and internal transcribed spacer (ITS) regions (Dresler-Nurmi *et al.* 1999). Two strains of *P. radiata* of Swedish and Finnish origins were the most closely related, based on restriction fragment analysis of the ITS regions. Interestingly, the four *P. tremellosa* isolates were indistinguishable, although these fungal strains were of Canadian and European origin (Dresler-Nurmi 1999).

4.2 Degradation of ^{14}C-(ring)-labelled DHP by *Phlebia* spp. (I)

^{14}C-ring-labelled synthetic DHP was mineralized by the well-characterized fungus *Phlebia radiata* 79 cultured under static and agitated conditions under an oxygen atmosphere. The shake flask cultures (25 ml) were supplemented with 0.05% (w/v) Tween 80 and 0.2 or 0.4 mM VA. They and the stationary flasks (10 ml, with 0.2 mM VA) were flushed with oxygen and cultures (10 ml) without inducer were incubated under a continuous oxygen stream (Fig. 4.5). The latter group without inducer attained the highest production of $^{14}CO_2$, almost 50% of total ^{14}C in 17 days. There was a very short lag stage (2 days) before the evolution of $^{14}CO_2$ began. In shake flask cultures with 0.2 mM VA, the fungus released 32% of the applied ^{14}C in 20 days, which was more than with higher VA (0.4 mM) level (27% as $^{14}CO_2$). The concentration of 0.2 mM VA was the most optimal level in lignin degrading cultures of *P. chrysosporium* in the studies of Lundquist and Kirk (1978). However, the supplementation of 0.2 mM VA did not increase $^{14}CO_2$ evolution under static conditions (Fig. 4.5).

The evolution of $^{14}CO_2$ by *Phlebia tremellosa* 77-51 from ^{14}C-(ring)-labelled DHP in basal medium was comparable to that of *P. radiata* cultures, although it was cultivated under different conditions (Fig. 6b in I). *Phlebia tremellosa* mineralized in the same time (20 days) about 32% of the total activity in standing cultures as *P. radiata* in shake flasks with 0.2 mM VA by flushing with oxygen. Mineralization of *P. tremellosa* remained lower when 50 x Mn(II) and 50 x Mn(II) plus malonate as chelator were added to the medium and compared with the basic manganese level. The high manganese concentration caused repression of $^{14}CO_2$ evolution, particularly during the first 2 weeks (Fig. 6b in I). The LiP activity was also repressed in bioreactors with a 10 x and 50 x Mn(II) content, pointing to the crucial role of LiP in ^{14}C-DHP mineralization by *P. tremellosa* strains (Fig. 1 in I).

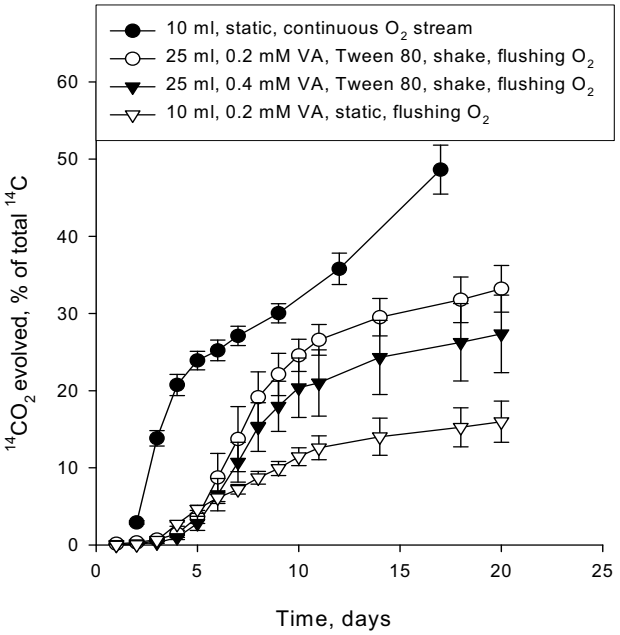


Fig. 4.5 Evolution of $^{14}CO_2$ from ^{14}C-(ring)-labelled DHP (the batch from K. Kirk) by *Phlebia radiata* 79 and influence of VA and Tween 80 detergent (0.05% w/v). ADMS-liquid media included LN (2 mM) and high glucose (HC, 1.0%). Six replicates were used, except in the static cultures without supplements (black circles), where four replicates were used.

The high concentration of Mn(II) with chelator could not stimulate mineralization in ^{14}C-(ring)-labelled DHP in white-rot fungal cultures. The regulative role of manganese in LiP production and lignin degradation by *P. tremellosa* is clear, but the mechanism behind this regulation is still unclear. The presence or absence of a chelator in cultures with high Mn(II) concentrations also did not affect the pattern of ^{14}C-DHP mineralization. The influence of Mn(II) on enzymatic activities and lignin degradation may be dependent on the differences in ligninolytic systems among the fungi (I).

4.3 Demethoxylation of lignin model compounds (II, III)

The white-rot fungi *Phlebia radiata* and *Phanerochaete chrysosporium* and the brown-rot fungi *Gloeophyllum trabeum* and *Poria placenta* were cultivated on solid agar media with a spruce wood block and without wood. The basal agar-ADMS medium contained nitrogen (2 mM) and glucose (0.1% LC or 1.0% HC) in different combinations. The 4-[$O^{14}CH_3$]-labelled dimer (veratrylglycerol-β-guaiacyl ether) or [$O^{14}CH_3$]-labelled vanillic acid were added at the same time as the inoculum on agar (in agar culture flasks) or on a wood block (in a wood block in the agar culture flask) at the beginning of the experiments. The demethoxylation activity was measured as $^{14}CO_2$ evolved from the fungal cultures.

4.3.1 White-rot fungi *P. radiata* and *P. chrysosporium* (II)

Agar cultures

Phlebia radiata 79 caused the highest $^{14}CO_2$ evolution, about 35% of the total ^{14}C in agar cultures with LN and HC, and with 2 mM nitrogen about 32% of the total ^{14}C under an air atmosphere in 8 weeks, but under oxygen the evolution of $^{14}CO_2$ was very poor (Fig. 2 in II). *Phanerochaete chrysosporium* evolved almost 35% of the $^{14}CO_2$ with LN and HC under an oxygen atmosphere, while in air without nutrient supplements the production was near 20% of the $^{14}CO_2$, but in the other growth medium group the production of $^{14}CO_2$ was weak (Fig. 3 in II).

Wood block cultures

With the two white-rot fungi *P. chrysosporium* and *P. radiata*, clearly the highest evolutions of $^{14}CO_2$ under an air atmosphere were seen with spruce wood when neither supplemented nitrogen nor glucose was present (Fig. 2-3 in II). Contrasting results were obtained under an oxygen atmosphere, where the lowest evolution of $^{14}CO_2$ was formed in cultures without nitrogen and glucose (Fig. 2–3 in II). In an oxygen atmosphere the LN and LN with glucose stimulated the production of $^{14}CO_2$. In the case of the other model compound, [O^{14}CH$_3$]-labelled vanillic acid, *P. radiata* evolved $^{14}CO_2$ from 38% to 48% during 9 weeks with birch wood under oxygen. The supplemented cultures, LN or LN + LC, weakly stimulated the release of $^{14}CO_2$ (Fig. 4 in II).

4.3.2 Brown-rot fungi *G. trabeum* and *P. placenta* (III)

The brown-rot fungus *Gloeophyllum trabeum* showed high demethoxylating activity on wood, which was higher than that from white-rot fungi. *Gloeophyllum trabeum* produced almost 60% $^{14}CO_2$ from ^{14}C-labelled compound in 8 weeks under an oxygen atmosphere with LN, which was the highest value measured as $^{14}CO_2$. During the same period, 55% of $^{14}CO_2$ from total ^{14}C was obtained with LN + HC. In an air atmosphere, evolved $^{14}CO_2$ by *G. trabeum* was similar (from 45% to 50%) in all three media groups (Fig. 1 in III). The evolution of $^{14}CO_2$ from [O^{14}CH$_3$]-labelled vanillic acid by *G. trabeum* was high, from 35% to almost 60% of the applied ^{14}C. LN plus LC provided the highest production of $^{14}CO_2$. The demethoxylating activity of *P. placenta* from the nonphenolic [O^{14}CH$_3$]-labelled *β*-O-4 dimer was poorer (3%) on birch hardwood than that from O^{14}CH$_3$-labelled vanillic acid, where it evolved $^{14}CO_2$ from 22% to 28% of the total ^{14}C (Fig. 2b, Table 1 in III). Davis *et al*. (1994) suggested that birch wood is degraded in a different way compared with spruce wood, and that the cleavage of the β-O-4 linkages in lignin was the most prominent reaction occurring, rather than demethoxylation of lignin by *P. placenta*. On the other hand, we used here different model compounds (O^{14}CH$_3$-labelled β-O-4-dimer and O^{14}CH$_3$-labelled vanillic acid) in the case of *P. placenta.*

The presence of wood increased the demethoxylation level of the lignin model compounds by the rot fungi, especially in the cultures of *G. trabeum* under air and the white-rot fungi *P. chrysosporium* and *P. radiata* under oxygen (Table 4.2). *Gloeophyllum trabeum* released 30–60% as $^{14}CO_2$ from the nonphenolic [O^{14}CH$_3$]-labelled β-O-4 dimer within 8 weeks under both atmospheres. The white-rot fungi attained similar levels in unsupplemented cultures under an air atmosphere.

Table 4.2 Demethoxylation of the nonphenolic [4-O$^{14}CH_3$]-labelled β-O-4 dimer with the brown-rot fungus *Gloeophyllum trabeum* and the white-rot fungi *Phanerochaete chrysosporium* and *Phlebia radiata* on solid agar and solid agar with softwood blocks (*Picea abies*). LN = low nutrient nitrogen (2 mM), LC = low glucose (0.1%) and HC = high glucose (1.0%). Data are means ± standard deviations of four replicates in the evolution of $^{14}CO_2$, and two parallels in weight loss samples. The growth times were 36–39 days.

		G. trabeum				*P. chrysosporium* and *P. radiata*			
Evolved $^{14}CO_2$%[a]	Wood present	no N, no C	LN, no C	LN, LC	LN, HC	no N, no C	LN, no C	LN, LC	LN, HC
O_2	yes	++	+++	nd	+++	++	++(+)	++(+)	++(+)
	no	++	+(+)	nd	+++	(+)	(+)	(+)	(+)
Air	yes	+++	+++	+++	nd	+++	++	nd	++
	no	++	++	++(+)	nd	+(+)	++	nd	++
[1]Weight loss from birch hardwood[b]		*G. trabeum*				*P. radiata*			
		++	++(+)	++(+)	nd	+	+(+)	+(+)	nd

[a]The symbols (+), +, +(+), ++, ++(+) and +++ indicate evolved $^{14}CO_2$% from total ^{14}C of < 10%, 10–20%, 20%, 20–30% 30–40% and > 50%, respectively.

[b]The symbols +, +(+), ++ and ++(+) indicate weight loss of 10–15%, 15–20%, 20–25% and 25–30%, respectively.

[1]Indicating cellulolytic activity

nd = not determined

Jin et al. (1990a) showed that *G. trabeum* evolves 30% from the applied ^{14}C-methylated lignin on pine wood flakes as $^{14}CO_2$. Haider and Trojanowski (1980) obtained 29% $^{14}CO_2$ from methoxyl-labelled DHP and 10% $^{14}CO_2$ from ring-labelled DHP with *G. trabeum* in 15 days. They also investigated demethoxylating and lignin-degrading activity with other brown-rot fungi and found wide variations between eight strains, which released 0.4–19.8% $^{14}CO_2$ from methoxyl-labelled DHP and 0.4–8.7% $^{14}CO_2$ from ring-labelled DHP in 15 days. Haider and Trojanowski (1980) concluded that brown-rot fungi strongly decrease methoxyl groups of lignin and increase carboxyl and carbonyl groups. *Gloeophyllum trabeum* showed the highest demethoxylating activities in the present cultures. An air atmosphere was at least as good as oxygen in these studies. *Gloeophyllum trabeum* also demethoxylated as high level in the cultures with LN and LC under an oxygen atmosphere, where it evolved nearly 60% as $^{14}CO_2$ from [O$^{14}CH_3$]-labelled vanillic acid.

4.4 Ergosterol as a measure of fungal growth (II, III, IV)

The amount of living biomass is a measure of the biological activity of microbial cells (Messner *et al.* 1998). Biomass has been considered one of the most important analytical parameters, e.g. in fermentation processes, because measurements of biomass enable control and optimization of the process (Messner *et al.* 1998). Metabolic activity is related to the growth of the organism, which makes it necessary to obtain a balance between the nutrients consumed and products formed (Singh *et al.* 1994). It is a simple matter to take samples from liquid cultures and weigh the amount of fungal biomass. In solid substrates, e.g. wood, this is not possible and indirect methods must be used. Background information and standardization are needed if the ergosterol amount is used to compare the growth of a certain fungus under different conditions, or to compare different fungi.

4.4.1 Ergosterol as an indicator of fungal biomass

Ergosterol is a frequently used indicator of fungal biomass, and abundant information is available on ergosterol in ascomycetes, but less on wood-rotting basidiomycetous fungi. The level of knowledge of ergosterol content in brown-rot fungi is poor, although they are common fungi and cause extensive damage to wood in service. The ergosterol concentration in fungal mycelia should be known, e.g. to follow the growth of a certain fungus under different conditions. Ergosterol is a good indicator and is considered a reliable measure of living fungal biomass when it is necessary to follow the growth of fungi in soil or solid cultivation media (Messner *et al.* 1998; Gessner and Newell 2002). Ergosterol contents in different type of cultures are summarized in Table 4.3.

4.4.2 Correlation of dry weight and ergosterol content of fungal liquid cultures (IV)

The dw of mycelia is used for the determination of biomass and is employed in the basic calibration of other methods, such as the ergosterol content (Singh *et al.* 1994; Messner *et al.* 1998; Gessner and Newell 2002). The ergosterol contents of the white-rot fungi *Phlebia radiata*, *Phanerochaete chrysosporium, Physisporinus rivulosus*, *Ceriporiopsis subvermispora,* and the brown-rot fungi *Gloeophyllum trabeum* and *Poria placenta* were analysed in liquid cultures (IV).

The ergosterol contents showed moderate correlation with dw ($r^2 = 0.384$, n = 30, each point is the mean of four replicates) in the case of these six fungi (Fig. 3 in IV). There were apparent relationships between ergosterol contents and mycelial dw in Fig. 4.6, when the fungi were analysed separately. The points of fungi are marked with distinct signs. *Physisporinus rivulosus* showed the highest correlation ($r^2 = 0.969$, n = 5) with dw but its ergosterol content showed the lowest value. The other selective white-rot fungus *C. subvermispora* showed strong correlation ($r^2 = 0.756$). The lowest correlation ($r^2 = 0.235$) was shown in the brown-rot fungus *G. trabeum*, while in the other brown-rot fungus *P. placenta* the correlation value was moderate ($r^2 = 0.392$). The correlations of *P. chrysosporium* and *P. radiata* were moderate, ($r^2 = 0.531$), and strong ($r^2 = 0.717$), respectively (Fig. 4.6).

Liquid cultures as standard cultures

Liquid cultures were also used as standard cultures for ergosterol assays of *P. chrysosporium* and *P. radiata,* cultivated in solid medium agar cultures with and without spruce wood blocks (Tables 2–5 in IV). The ergosterol contents of six fungi and ergosterol

Table 4.3 Ergosterol contents from different fungi (dw = dry weight).

Fungi	Sample	Ergosterol contents /in time	Reference
Three *Aspergillus glaucus* group	Liquid cultures	2.3-5.9 mg g^{-1}dw/ 2-10 days	Seitz *et al.* 1979
Mycorrhizal fungi	Pure culture	2.88-3.59 mg g^{-1}/ 4 weeks	Salmanowicz & Nylund 1988
Ophiostoma piceae	Liquid cultures	5 mg ml^{-1} dw/ 5 days	Gao *et al.* 1993
Ophiostoma piceae	Blocks of lodgepole pine wood	32 µg g^{-1} dry wood/ 7 days	Gao *et al.* 1993
Ophiostoma piliferum	Aspen wood chips in *O. piliferum* Cartapip™ 97	33 µg g^{-1} dry wood/ 15 days	Gao *et al.* 1993
Four *Lentinus edodes* strains, *Lentinus lepideus*	Submerged liquid culture	0.124-0.24 mg g^{-1} dw 0.695 mg g^{-1} dw	Okeke *et al.* 1994
Ceriporiopsis subvermispora	Wood chips in corn steep liquor cultures	0.08 mg g^{-1} wood/ 35 days	Messner *et al.* 1998
Filamentous fungi and yeasts	Pure broth cultures	2.6-14 and 37-42 mg g^{-1} dry mass respectively	Pasanen *et al.* 1999
Six fungi isolated from soil and plant materials	Cultured in three different broth media	4 µg mg^{-1} dry biomass/ 3-10 days	Montgomery *et al.* 2000
Aquatic hyphomycetes fungi	Liquid cultures	10-11 µg mg^{-1} dry mass	Charcosset & Chauvet 2001
20 white-rot fungi[1)]	Liquid cultures	2.38-13.06 mg g^{-1} dry biomass/ 7 days	Barajas-Aceves *et al.* 2002
Agaricus bisporus/white and brown, *Pleurotus ostreatus, Lentinus edodes*	Edible cultivated mushrooms	6.02-6.78 mg g^{-1} dw	Mattila *et al.* 2002
Chantarellus cibarius, C. tubaeformis, Boletus edulis, Lactarius trivialis	Edible wild mushrooms	2.96-4.89 mg g^{-1} dw	Mattila *et al.* 2002
Paxillus involutus, Suillus bovinus, Suillus variegatus	Growth in pure culture on agar	1.8-5.4 mg g^{-1}	Ohlsson *et al.* 2003

11 fungi, incl. *Schizophyllum commune, Phanerochaete chrysosporium*	Potato dextrose agar media	3.2 and 4.9 mg g^{-1} mycelium dw/ 12 and 24 days	Klamer and Bååth 2004
Trametes versicolor	Birch wood (*Betula pubescens*)	30.2 µg g^{-1} wood/16 weeks	Eikenes *et al.* 2005
Phlebia radiata and *Phanerochaete chrysosporium*	Spruce wood (*Picea abies*) on solid agar cultures	15-43 µg g^{-1} dw/ 14 days 20-50 µg g^{-1} dw/ 35 days	Niemenmaa *et al.* 2006 (II)
Phlebia radiata and *Phanerochaete chrysosporium*	Agar cultures	150-540 µg g^{-1} dw/ 14 days 80-356 µg g^{-1} dw/ 35 days	Niemenmaa *et al.* 2006 (II)

[1)] *Coriolopsis gallica*, *Ganoderma applanatus*, *Trametes versicolor* (2), *Sporotrichum pruinosum*, *Phanerochaete chrysosporium* (4), *Pleurotus ostreatus* (8), *Bjerkandera adusta* (3)

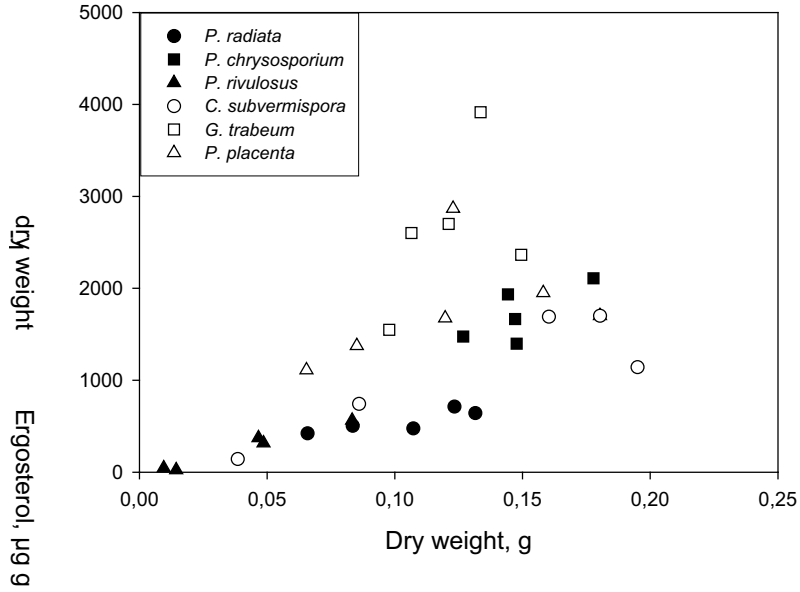


Fig. 4.6 Relationship between egrosterol contents and mycelial dry weights from liquid cultures. The ergosterol contents were calculated per fungal dry weights from 1 to 5 weeks. The fungi were cultivated in ADMS medium with low nitrogen (2 mM) and low glucose (0.1%). Four parellel samples were used for each data point.

as percentage of dw are illustrated in IV (Table 1). Those values calculated as percentage of dw are demonstrated in the same figure (Fig. 2 in IV). The curves show less strong variation between weeks than the ergosterol values alone. The brown-rot fungi *G. trabeum* and *P. placenta* showed the highest ergosterol contents as percentage dw. The white-rot fungi *P. chrysosporium* and *P. radiata* produced biomasses in which ergosterol was most constant. The content of ergosterol as percentage of dw increased constantly with time in the other selectively wood-degrading fungus, *P. rivulosus*, although its ergosterol contents were low, but in *C. subvermispora* the fraction of ergosterol varied widely during the cultivation time.

4.4.3 Ergosterol as a measure of growth (II, III, IV)

Wood-block cultures

The ergosterol contents were measured from fungal cultures of *P. radiata* 79 and *P. chrysosporium* 1767 grown in spruce wood blocks on solid agar medium (Fig. 4.7). The amounts of ergosterol in these cultures were stable with time, although in *P. radiata* the ergosterol amounts gradually increased towards the end of the growing time (Fig. 4.8). The highest ergosterol contents were obtained in LN-supplemented cultures (2 mM) without glucose (Tables 3 and 4 in IV, Fig. 4.8). Wood appeared to compensate for glucose in both fungi, *P. radiata* and *P. chrysosporium*, probably due to the storage nutrients in wood that may play an important role in growth. The fungi did not use the available glucose, because the wood may have provided other carbon nutrients (wood polysaccharides) more suitable than glucose for the fungi.

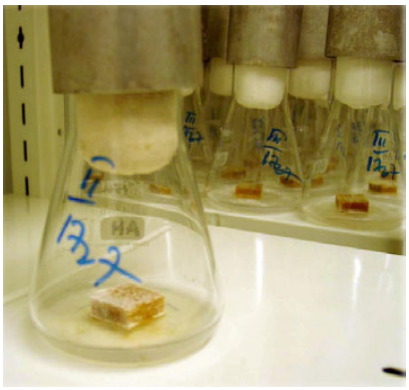


Fig. 4.7 a, b. Spruce wood block on agar culture flasks for ergosterol analysis on day 6 in incubation room at 28 oC.

The cultures in ^{14}C-labelled *β*-O-4 dimer by *P. radiata* differed from the above, in that the demethoxylation activity was highest under air when no supplements were present. However, in an oxygen atmosphere the highest release of $^{14}CO_2$ was obtained with nitrogen and glucose supplements (Figs. 2 and 3 in II). Messner *et al*. (1998) showed that added soluble nutrients do not diffuse into wood chips. They suggested that the supplemented nutrients help to initiate fungal growth on the surface of the chips, which increases the biomass and enables colonization in the deeper area of the wood chip.

Agar cultures

The ergosterol contents in the cultures of *P. radiata* 79 and *P. chrysosporium* 1767 were analysed weekly from days 7 to 35 in the agar cultures. The ergosterol contents of both fungi were highest (about 650 µl g^{-1} and 400 µl g^{-1} dw) in the culture medium group, with 2 mM nitrogen and LC with *P. chrysosporim* and *P. radiata*, respectively (Tables 4 and 5 in IV). This medium combination was nutritionally analogous to the medium of liquid cultures, where six fungi were grown (Table 1 in I). The nutrients were depleted, especially in the flasks without nitrogen and glucose, because there were no other carbon or nitrogen sources. In the experiments with nonphenolic [O^{14}CH$_3$]-labelled β-O-4 dimer, the *P. radiata* behaved similarly without wood (Fig. 2 in II), when the highest activity was also seen in the cultures with LN and glucose. The amount of ergosterol decreased in the LC concentration cultures under prolonged incubation time (Bjurman 1994), but in wood cultures at earlier growth phases higher ergosterol content were obtained with LC. Bjurman (1994) also found higher ergosterol contents with enriched nitrogen or malt extract cultures.

4.4.4 Conversion factor in measurements of biomass

Information on ergosterol content alone does not give the absolute biomass amount of the fungus and therefore appropriate factors are needed to convert ergosterol values into mycelial biomass (Gessner and Chauvet 1993). These measurements mostly concern soil-originating and soft-rot fungi, e.g. *Ascomycetes* and mitosporic fungi, as well as aquatic hyphomycete fungi. Gessner and Chauvet (1993), Montgomery *et al.* (2000) and Gessner and Newell (2002) discussed the use of conversion factors as indicators of biomass in fungal cultures. Several terms, such as mycelial dry mass, dw or species-specific value, have been used to describe biomasses. There are many precautions in the use of conversion factors (ergosterol to biomass). The culturing conditions must be chosen so that standard growth

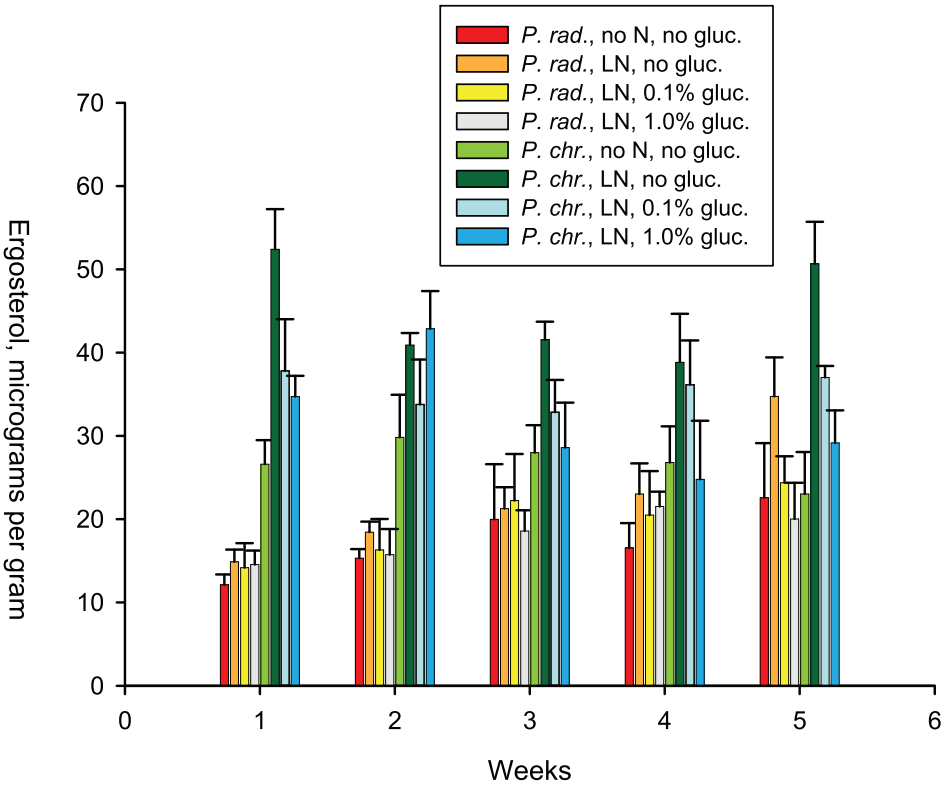


Fig. 4.8 Ergosterol contents of *P. radiata* and *P. chrysosporium* cultures in wood (spruce) block with different ADMS-medium combinations. ADMS-agar-medium was not supplemented or supplemented by low nitrogen (LN) or by LN with low (0.1%) or high (1.0%) glucose. The first four columns demonstrate *P. radiata* and the last four columns *P. chrysosporium* in each week. The standard deviations are from four parallel cultures.

conditions closely resemble those of the cultures to be compared (Gessner and Chauvet 1993). Bjurman (1994) pointed out that ergosterol is related to the amount of viable mycelia and that conversion factors relating to biomass are highly variable.

Gessner and Chauvet (1993) and Montgomery *et al.* (2000) calculated conversion factors for biomasses according to the equation: dw [1 g] / ergosterol mg g^{-1} x 1000. Montgomery *et al.* (2000) obtained ergosterol 4 µg mg^{-1} biomass (i.e. 0.4%), and a conversion factor of 250 as an average of six fungi from soil. The ergosterol contents varied from 0.4% to 1.0%. Gessner and Chauvet (1993) analysed the ergosterol content in 13 aquatic hyphomycete fungi and calculated that the general conversion factor was 182 and that there were 5.5 mg ergosterol g^{-1} of dry mass. Messner *et al.* (1998) showed that in *C. subvermispora* an ergosterol content of 0.7% correlated with a fungal biomass of 5 mg g^{-1} wood. Gessner and Chauvet (1993), Ruzicka *et al*. (2000), Charcosset and Chauvet (2001) and Klamer and Bååth (2004) used means of 5 or 5.5 mg ergosterol g^{-1} dw of fungal mycelium, from which the conversion factor was derived. This value was based on the results of various fungi originating from soil, compost and aquatic hyphomycete fungi cultivated in liquid cultures. Klamer and Bååth (2004) noted that the use of 5 mg g^{-1} dw can in some cases give

impossible results. They calculated the conversion factors for different types of compost-originated fungi to estimate the fungal biomass by quantification of PLFAs to ergosterol contents (1 mg ergosterol g^{-1} biomass C and 11.8 μmol 18:2 ω 6,9 biomass C).

4.4.5 Use of conversion factors in wood block and agar cultures

The conversion factors calculated from liquid (standard) cultures were used in calculating ergosterol contents in agar and wood block cultures (Table 6 in IV). We used conversion factors where dw of fungi [mg] is divided by the value for ergosterol [μg g^{-1}] and multiplied by 1000 (Tables 6 and 7 in IV). Calculation of conversion factors by Gessner and Chauvet (1993) and Montgomery *et al*. (2000) would have given unfeasible results and therefore here a modification of their conversion factor was used (Table 6 in IV). The conversion factor for *P. chrysosporium* was 88 and for *P. radiata* 185. They were taken into account in calculating the biomasses in wood block and agar cultures and in culture medium groups, which were supplemented with 2 mM-N and LC, the same combination as in liquid cultures (Table 7 in IV). It must be realized that although a conversion factor can be calculated, it can still be an arbitrary value. In using conversion factors the same trends were apparent, but the values were 5.5 (*P. radiata*) and 11 (*P. chrysosporium*) times higher than the measured original values (Table 7 in I). The theoretical calculated conversion factors are always somehow arbitrary counts that likely gave underestimated results. Gessner and Newell (2002) proposed that the use of conversion factors can lead to a two- to threefold over- or underestimation of the true fungal biomass.

Gao *et al.* (1993) found in liquid media a good correlation between fungal biomass measured as dw and ergosterol amount. They showed that it is inaccurate to convert ergosterol values to direct fungal biomass, since the ergosterol content is not constant during different fungal growth stages. Singh *et al*. (1994) and Matcham *et al.* (1985) suggested that the ergosterol content showed a relationship similar to that of mycelium growth in liquid culture, but that with solid substrates the relationship was unsatisfactory.

Ergosterol is a commonly used indicator for fungal biomass, but little information is available on wood-rotting basidiomycetous fungi. Taxonomically, these rather closely related fungi can differ widely in their ergosterol contents under the same culture conditions. Ideally a conversion factor of ergosterol should be determined from standard cultures of each fungus, if possible so that the fungi are cultured under the same conditions and ergosterol analysed in the same way. Samples should be taken quite often, because each fungus behaves individually, making the entire process laborious and difficult to put into practice. If the ergosterol contents are compared between different sampling days, it would be come a useful method that would complement other methods, such as enzyme assays and radiorespirometric techniques, in measurements of fungal growth.

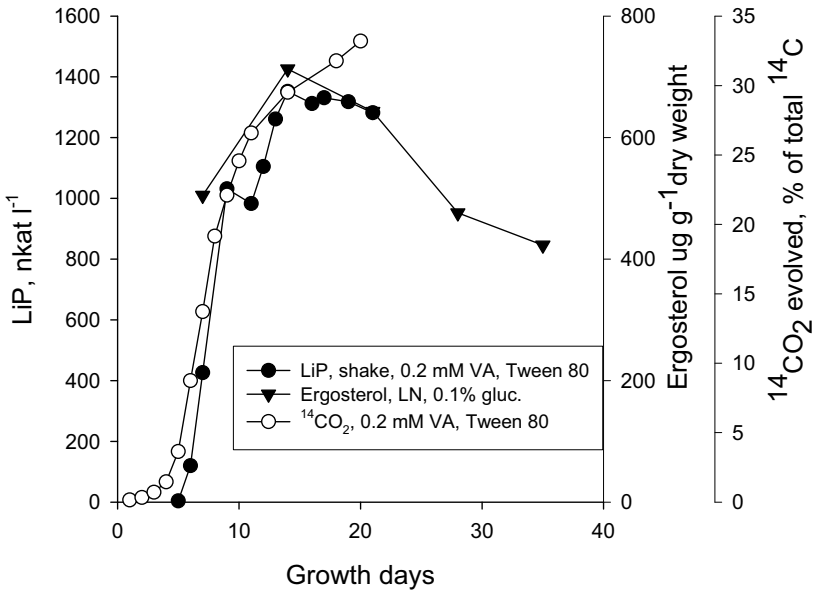


Fig. 4.9 *Phlebia radiata* 79 was grown in shake flasks (25 ml), supplemented with 0.2 mM VA and Tween 80 (0.05%) for LiP, in nonagitated flasks (10 ml) for ergosterol analysis, with low nitrogen (2 mM) and glucose (0.1%) and in ^{14}C-(ring)-labelled DHP cultures (25 ml) under agitation and flushed with oxygen, with 0.2 mM VA and Tween 80 (0.05%).

4.4.6 *Phlebia radiata* monitored by different methods

The white-rot fungus *Phlebia radiata* 79 was present in all the experiments conducted and here its growth in the various methods used is compared (Fig. 4.9). The production of LiP from days 4 to 22 correlated very well with degradation of ^{14}C-(ring)-labelled DHP cultures as $^{14}CO_2$ quantified from 1 to 20 days. The ergosterol contents were analysed during 5 weeks and the results obtained supported those of LiP and $^{14}CO_2$. However, the maximum level of biomasses as ergosterol could not be observed, because the ergosterol contents were determined only once per week. Overall, the most intensive growth of fungus was determined both by degradation of ^{14}C-(ring)-labelled DHP and LiP the results of which were similar between days 7 and 9. The ergosterol contents correlated with these findings, although the highest point was at day 14.

5 SUMMARY

Using different monitoring techniques, new fungi can be found for ecological and biotechnical purposes. Here, we studied in further detail the growth of white-rot fungi, e.g. the selectively lignin-degrading *Phlebia radiata, Phlebia tremellosa*, *Ceriporiopsis subvermispora* and *Physisporinus rivulosus*, which previously were the most promising fungi for biopulping - cultivation of fungi on wood chips prior to mechanical pulping to save refining energy. The lignin-degrading ability of fungi was analysed by measurement of $^{14}CO_2$ from ^{14}C-labelled compounds, which is a reliable method for demonstrating fungal activity. The evolution of $^{14}CO_2$ from ^{14}C-labelled compounds correlated well with the production of LiP enzymes. Development of $^{14}CO_2$ from the labelled methoxyl compounds by brown-rot fungi, e.g. *Gloeophyllum trabeum* and *Poria placenta*, is evidence of their capability to demethoxylate lignin model compounds. In many cases, ligninolytic enzymes produced under solid cultivation conditions are studied, although measurements of enzyme activity in this type of sampling are challenging. Determining the ergosterol content may enable indication of fungal activity in solid substrates, although it is a complicated procedure.

The *Phlebia* strains showed similarities with their enzyme profiles: all of them produced 2–3 LiPs and one laccase. *Phlebia radiata* 79 (the Finnish strain) and *P. radiata* L12-41 (the Swedish strain) also produced two MnPs. *P. tremellosa* 2845, the Canadian strain, resembled the *P. tremellosa* strains (76-24, 77-51, 79-16) received from Estonia. They showed MnP activity, although the enzyme could not be purified. The Estonian P. *tremellosa* strains mineralized ^{14}C-(ring)-labelled DHP as well as *P. radiata* and *P. chrysosporium* under the same culture condition. High manganese concentrations suppressed mineralization of ^{14}C-(ring)-labelled DHP, even with the chelator sodium malonate, showing that manganese participates in regulation of lignin degradation.

The demethoxylation of the phenolic [$O^{14}CH_3$]-labelled β-O-4 dimer was higher in spruce wood cultures than that without wood both in *P. radiata* and *P. chrysosporium* and the brown-rot fungus *G. trabeum*. An air atmosphere was at least as good as an oxygen atmosphere in evolvement of $^{14}CO_2$ from [$O^{14}CH_3$]-labelled β-O-4 dimer. With *P. radiata* and *P. chrysosporium* the highest demethoxylation activity occurred under air without nitrogen and glucose supplements when wood was present. In *G. trabeum* the demethoxylation was strongest with nitrogen or nitrogen plus glucose with wood, both under air and oxygen atmospheres. The glucose and nitrogen nutrients also increased demethoxylation from [$O^{14}CH_3$]-labelled vanillic acid on birch wood by *P. radiata* and the brown-rot fungi *G. trabeum* and *P. placenta*.

The highest ergosterol contents were obtained in liquid cultures of the brown-rot fungi *G. trabeum* and *P. placenta*. The level of ergosterol was most stable during culturing time with *P. radiata* and *P. chrysosporium*. The variation was highest in the cultures of *C. subvermispora*, while in *P. rivulosus* the ergosterol contents were low but increased constantly during the following weeks. The ergosterol contents and dw correlated well in all fungi. In solid agar cultures of *P. radiata* and *P. chrysosporium*, the wood blocks stabilised the biomass production, while without wood, the ergosterol contents were highest at the beginning of cultivation, but decreased with decrease in nutrients.

6 CONCLUSIONS

The main conclusions were:

1 The strains of *Phlebia radiata* and *Phlebia tremellosa* were similar in their enzyme profiles; all produced 2–3 LiPs, while the *P. radiata* strains also produced two MnPs. *Phlebia tremellosa* strains (2845, 76-24, 77-51, 79-16) showed MnP activity, but the enzyme protein could not be purified. All *Phlebia* spp. showed laccase activity.

2 The mineralization rates of ^{14}C-(ring)-labelled DHP by three Estonian *P. tremellosa* strains under basal Mn(II) level were similar to those obtained with *P. radiata* 79 and *Phanerochaete chrysosporium*.

3 A high-manganese (50x Mn(II) concentration) repressed the evolution of $^{14}CO_2$ from ^{14}C-(ring)-labelled DHP even when the chelating agent, sodium malonate, was included in the medium. The LiP activity was also repressed with a 10x and 50x Mn(II) content, pointing to the crucial role of LiP in the degradation of synthetic lignin.

4 The highest production of $^{14}CO_2$ was obtained from the [$O^{14}CH_3$]-labelled dimeric lignin model compound by white-rot fungi *P. radiata* and *P. chrysosporium* and brown-rot fungus *Gloeophyllum trabeum* in medium with no supplements (nitrogen or glucose) under air in the presence of wood. Wood clearly compensated for the lack of nutrient nitrogen and glucose.

5 An air atmosphere was at least as good as an oxygen atmosphere in the demethoxylation of ^{14}C-labelled compounds in the cultures on wood blocks both by the brown-rot fungus *G. trabeum* and the white-rot fungi *P. chrysosporium* and *P. radiata.*

6 Wood in solid agar cultures stabilized the cultures of *P. radiata*, while the biomass measured as ergosterol content increased slightly to the end of cultivation. In the agar cultures the ergosterol contents were highest at the beginning of cultivation.

7 In the wood block cultures of *P. chrysosporium* and *P. radiata* the highest biomass (based on ergosterol assay) was obtained with LN but without glucose. The highest biomass in agar cultures was obtained with a combination of LN plus LC.

8 Measurements of ergosterol can be considered a useful method for measuring biomass in fungal culture, although it is rather laborious.

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